Development of a protocol for the rapid *in vitro* establishment of *Eucalyptus* clones

by

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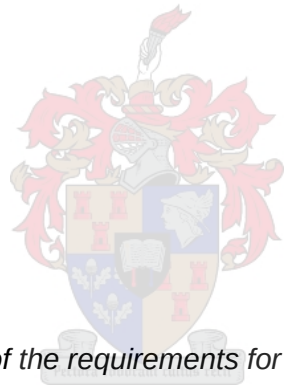

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Sciences at Stellenbosch University.

This thesis is prepared in accordance to the format of Southern Forests, but it is more elaborate for the purposes of the present dissertation. The manuscript will be appropriately reduced and submitted for peer review once permission has been obtained from the forestry company whom supplied the Eucalyptus *clones utilised in this research, under the terms of the Materials Transfer Agreement.*

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

The practice of cultivating *Eucalyptus* species using micropropagation has found favour in the development of successful forestry plantation programs due to its effectiveness in generating large numbers of juvenile plant stocks. Natural phytohormones and their synthetic analogues, called plant growth regulators (PGRs), are typically included in growth media during micropropagation. These compounds may be used to stimulate certain physiological processes in plants, allowing, for example, the *in vitro* manipulation of plant cell growth and differentiation, depending on the concentrations and combinations of specific PGRs in the medium. The development of micropropagation programs for *Eucalyptus* spp*.* are often clone-specific, necessitating optimisation for each clone. There is thus an inherent need for the development of a standardized protocol permitting the *in vitro* establishment and proliferation of multiple *Eucalyptus* clones.

In the present study, the sensitivity and responsiveness of three *Eucalyptus grandis* × *Eucalyptus nitens* clones to various PGRs were investigated for the different *in vitro* growth stages. They include auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA). Similarly, the cytokinins kinetin, 6-benzylaminopurine (BAP) and *meta*-topolin were tested. The effects of strigolactones on micropropagation were examined using the synthetic strigolactone analogue, GR24. Parameters such as explant multiplication, shoot elongation and rooting were examined for all clones. The explants were incubated on a reduced PGR maintenance medium, from which source material was derived for subsequent experimentation, enabling the growth stages to be investigated independently. The highest level of bud initiation and shoot growth was observed for all *Eucalyptus* clones when the growth medium was supplemented with 0.5 mg ℓ^{-1} *of meta*-topolin in combination with 0.1 mg ℓ^{-1} of IAA. Optimal elongation for all the clones was detected upon addition of *meta*-topolin at 0.05 mg ℓ^{-1} and IAA at 0.5 mg ℓ^{-1}. The peak rooting response was obtained with 0.5 mg ℓ^{-1} of IBA for clones 2 and 3, whilst 0.029 mg ℓ^{-1} of GR24 in combination with 0.5 mg ℓ^{-1} of IAA elicited optimal rooting for clone 1. The most consistent rooting response across all clonal lines, however, was obtained with the GR24 treatment.

Clone 2 was found to be the most responsive to *in vitro* stimulation by auxin and this observation was further probed via RT-qPCR. Expression of the genes encoding the auxin efflux and influx transporters PIN1 and AUX1, respectively, were analysed to assess whether clonal response could be linked to expression of a particular gene(s). Equal expression levels of *PIN1*:*AUX1* were detected for clone 2. Clones 1 and 3, however, exhibited an expression profile whereby *PIN1* transcript levels exceeded those of *AUX1* when treated with 0.5 mg ℓ^{-1} of IAA. These expression profiles suggest that equivalent expression of *PIN1:AUX1* correlated with greater responsiveness to exogenously supplied IAA for clone 2. The findings of this study thus suggest a novel approach for the rapid determination of *in vitro* responses of valuable *Eucalyptus* genotypes, by investigating culture growth stages and gene expression levels.

Samevatting

Die gewoonte om *Eucalyptus*-spesies deur mikrovoortplanting te kweek het guns gevind in die ontwikkeling van suksesvolle bosbou-plantasieprogramme vanweë die doeltreffendheid daarvan om menige jeugdige plantvoorrade te genereer. Natuurlike fitohormone asook hul sintetiese analoë, genaamd plantgroeireguleerders (PGRs), word tipies in groeimedia ingesluit tydens mikrovoortplanting. Hierdie samestellings mag gebruik word om sekere fisiologiese prosesse in plante te stimuleer wat, by voorbeeld, die *in vitro*-manipulering van plantselgroei en-differensiasie toelaat, afhangende van die konsentrasies en kombinasies van spesifieke PGRs in die medium. Die ontwikkeling van mikrovoortplantingsprogramme vir *Eucalyptus* spp. is dikwels kloonspesifiek, wat optimalisering vir elke kloon vereis. Daar bestaan dus ‘n inherente behoefte aan die ontwikkeling van ‘n gestandaardiseerde protokol wat die *in vitro*-vestiging en-vermenigvuldiging van veelvuldige *Eucalyptus*-klone toelaat.

In die huidige studie was die sensitiwiteit en responsiwiteit van drie *Eucalyptus grandis* × *Eucalyptus nitens* klone teenoor verskeie PGRs ondersoek vir die verskillende *in vitro*-groeistadiums. Dit sluit ouksiene in soos indool-3-asynsuur (IAA), indool-3-bottersuur (IBA) en 1-Naftaleen-asynsuur (NAA). Desgelyks was die sitokiniene kinetien, 6-Bensielaminopurien (BAP) en *meta*-topolien getoets. Die uitwerkings van strigolaktone op mikrovoortplanting was ondersoek deur aanwending van die sintetiese strigolaktoon-analoog, GR24. Parameters soos eksplantvermenigvuldiging, skootverlenging en wortelskieting was vir alle klone ondersoek. Die eksplante was op ‘n verlaagde PGR-instandhoudingsmedium geïnkubeer, waarvan bronmateriaal afgelei is vir daaropvolgende eksperimentering wat die onafhanklike ondersoek van groeistadiums moontlik gemaak het. Die grootste vlak van knoppie-inisiëring en skoot-ontwikkeling was waargeneem vir alle *Eucalyptus*-klone toe die groeimedium aangevul is met 0.5 mg ℓ^{-1} *meta*-topolien in kombinasie met 0.1 mg ℓ^{-1} IAA. Optimale verlenging van al die klone was gewaar met toevoeging van *meta*-topolien by 0.05 mg ℓ^{-1} en IAA by 0.5 mg ℓ^{-1}. Die piekwortelrespons was verkry met 0.5 mg ℓ^{-1} IBA vir klone 2 and 3, terwyl 0.029 mg ℓ^{-1} GR24 in kombinasie met 0.5 mg ℓ^{-1} IAA optimale wortelskieting vir kloon 1 ontlok het. Die

mees konsekwente wortelskietrespons, oor alle klonale lyne, was egter met die GR24-behandeling behaal.

Daar was gevind dat kloon 2 mees responsief is op *in vitro*-stimulasie deur ouksien en hierdie waarneming was verder ondersoek via RT-qPCR. Uitdrukking van die gene wat onderskeidelik die ouksien-uitvloei- en invloeivervoerders PIN1 en AUX1 kodeer was geanaliseer om te bepaal of klonale vatbaarheid gekoppel kan word aan die uitdrukking van ‘n spesifieke geen(e). Gelyke uitdrukkingsvlakke van *PIN1*:*AUX1* was vir kloon 2 bespeur. Klone 1 en 3 het egter ‘n uitdrukkingsprofiel getoon waardeur *PIN1*-transkripvlakke dié van *AUX1* oorskry het tydens behandeling met 0.5 mg ℓ^{-1} IAA. Hierdie uitdrukkingsprofiele suggereer dat ekwivalente uitdrukking van *PIN1:AUX1* ooreenstem met groter responsiwiteit op eksogene verskafde IAA vir kloon 2. Die bevindinge van hierdie studie stel dus ‘n nuwe benadering voor vir die vinnige bepaling van *in vitro*-reaksies van waardevolle *Eucalyptus*-genotipes, deur die ondersoek van kultuur groeistadiums en geenuitdrukkingvlakke.

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List of Abbreviations and non-SI units

Abbreviation	Expansion
ABP1	Auxin binding protein 1
ACT2	Actin 2
AUX1	Auxin transporter protein 1
BAP	6-benzylaminopurine
bp	Base pair
°C	Degrees Celsius
Ca	Calcium
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
cm^3	Centimetre cubed
CTAB	Cetyltrimethylammonium Bromide
d	Day
ddH2O	De-ionised distilled water
dH20	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Et	Elongation treatment
Fig	Figure
Fp	Forward primer
$g\ell^{-1}$	Grams per litre
h	Hour
ha	Hectares
$HgCl_2$	Mercuric chloride
H2B	Histone 2 B
$kgf\ cm^{-2}$	Kilogram force per square centimetre
IAA	Indole-3-acetic acid

IBA	Indole-3-butyric acid
IPB	Institute for Plant Biotechnology
M	Molar
Mg	Milligram
mg ℓ^{-1}	Milligram per litre
min	Minute
mℓ	Millilitre
mM	Millimolar
MS	Murashige and Skoog (basal medium)
$m^{-2}s^{-1}$	Square metres per second
Mt	Multiplication treatment
NAA	1-Naphthaleneacetic acid
NaOCl	Sodium hypochlorite
ng	Nanogram
No	Number
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PVC	Polyvinyl chloride
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
Rp	Reverse primer
rRNA	Ribosomal ribonucleic acid
Rt	Rooting treatment
RT-sqPCR	Semi-quantitative reverse-transcriptase-PCR
s	Second
TDZ	Thidiazuron
T_m	Melting temperature
TUA	Tubulin alpha chain
TZ	Trans-zeatin

U$\mu\ell^{-1}$	Units per microliter
UV	Ultraviolet
V	Volt
wv^{-1}	Weight per volume
x*g*	Relative gravitational force
YUC3	Yucca 3
ZAR	South African Rand
Zn	Zinc
&	And
$\bar{x}$	Mean
µg	Microgram
µg $m\ell^{-1}$	Microgram per millilitre
µm	Micrometre
µℓ	Microlitre

CHAPTER I

Introduction and Literature review

The importance of natural forests

Trees comprise a significant proportion of the Earth's biomass and, primarily through carbon fixation, play a crucial role in supporting all terrestrial life forms. Humans depend on trees as sources of food, shelter and fuel (Trumbore et al. 2015). The global demand for forestry resources has placed significant pressure upon indigenous forest ecosystems. These ecosystems comprise of a multitude of fauna and flora species, many of which are endemic and adapted to sensitively balanced ecosystems. However, the felling of indigenous forests to meet the demand for timber and the commercial viability of the enterprise threatens the fragile balance of these ecosystems (Cummings et al. 1997). In 2015, The United Nations Food and Agriculture Organisation (2015) reported that an estimated net loss of 129 million hectares of natural forests occurred during the course of the 25 years, from 1990 to 2015 (FAO 2015). The establishment of well-managed commercial forest plantations is thus considered a viable and sustainable alternative to meet global timber demands and ease native deforestation.

South Africa is generally considered to be a semi-arid country, with the evaporative demands greatly exceeding the mean annual precipitation. Consequently, the majority of the vegetation in South Africa comprises of non-woody plant species (DWAF 2004; Schulze 2008; Scott and Gush 2017). South African native forests consist of open canopy savannah forests in the north-eastern interior of the country as well as closed canopy woodlands along the southern and eastern coastlines. The South African savannah forests covers approximately 19.0% of the entire land area, followed by the closed canopy forests covering approximately 0.5% of the land, figures which are well below the world mean for indigenous forests, which is in excess of 30% (Owen and Zel 2000). South

African indigenous forests display slow growth rates and thus are difficult to re-generate. As a result, the majority of these forest areas are not used for commercial production, but are instead managed for conservation purposes (Sedjo 1999; Scott and Gush 2017).

Commercial forests in South Africa are dominated by *Pinus* (pines), *Eucalyptus* (eucalypts) and *Acacia* (wattles) species, originating from South-eastern USA and Australia, respectively. In 2007, approximately 54% of the plantations in South Africa consisted of pines and other softwood species, whilst 46% of the plantations comprised of the hardwood species eucalypts and wattle (FSA 2007). Due to the desirable growth characteristics observed in eucalypts, 42.7% of the total plantation area in South Africa in 2016 consisted of *Eucalyptus* species, cultivated for their wood and related forestry products (Godsmark and Oberholzer 2017).

Eucalyptus *history and use in silviculture*

The genus *Eucalyptus* is considered to be among the largest of flowering trees worldwide (Leslie and Purse 2016). This genus is counted amongst some of the most widely propagated hardwood species in the world, which include Poplar and Pine (Mott et al. 2010; Lovarelli et al. 2018). Although eucalypts comprise of well over 700 species, *Eucalyptus* silviculture is dominated by relatively few species, namely *E. camaldulensis*, *E. grandis*, *E. dunii*, *E. nitens*, *E. urophylla, E. saligna, E. globulus*, *E. pellita* and *E. tereticornis* (Stanturf et al. 2013). According to Mc Mahon et al. (2019), approximately 20 million hectares of land is cultivated for *Eucalyptus* plantations globally, which may be attributed to a host of factors that includes the adaptability of this genus to a variety of climatic and soil conditions, desirable hardwood characteristics, fast growth rates and the multitude of products such as timber, pulp, essential oils, bioactive chemicals and biofuels that can be produced directly or indirectly from eucalypt forests (Turnbull 1999; Palett and Sale 2004; Hinchee et al. 2009). Given that eucalypts are indigenous to Australia, *Eucalyptus* species acquired a high degree of adaptability in response to the separation of Australia from Antarctica some 30 million years ago. The geographical event resulted in a rapidly changing climate in what is now Australia, which became progressively dryer

and hence the eucalypts of the time were required to adapt to this rapidly dynamic environment (Hill et al. 1999; Myburg et al. 2007). Consequently, eucalypts have developed into multiple species with a diverse gene pool, resulting in a genus that is suited to a range of climates worldwide, and as such, these trees are considered the perfect source of primary raw material for commercial forestry products. During colonisation by European settlers, traders, botanists and soldiers, eucalypts were dispersed to many countries, including the Americas, South Africa and Asia (Nakhooda and Jain 2016). *Eucalyptus* gained popularity via private breeders and botanical gardens in the mid to late nineteenth century (Benett 2010). Various species of eucalypts were introduced in this way to countries such as South Africa, India and Thailand (Baltimore 2000). In South Africa the potential for eucalypts to contribute to the economy and agriculture was quickly recognised, and towards the late nineteenth to early twentieth century market-based and capital-intensive plantations were established (Benett 2010). Initially there was resistance to the introduction of these trees into South Africa from environmental and national lobbies, who believed that *Eucalyptus* plantations would interfere with the native South African flora (Smuts 1968). Yet, despite the attempts to prevent further cultivation of these species, eucalyptus timber export grew, significantly contributing to the South African economy (Rotberg 1980). *Eucalyptus* spp. have commonly been hybridised to obtain characteristics suited to a particular environment or purpose (Butcher et al. 2005; Bennett 2010). For example, studies conducted by Brondani et al. (2011) utilised a *E. benthamii x dunnii* hybrid, combining the cold-tolerance of *E. benthamii* with the fast growth rates and desirable hardwood properties of *E. dunnii*, allowing for optimal growth in a vast array of environments (Jovanovic et al. 2000; Butcher et al. 2005).

Economic impact of Eucalyptus *on the South African forestry industry*

In South Africa, *Eucalyptus* plantations have provided a cost effective and renewable source of raw materials which have typically been utilised for railway sleepers, poles, firewood, ornaments, furniture and more recently, in large scale paper and pulp industries (Turnbull 1999; Watt 2013). Eucalypts were a preferred source over indigenous species

due to low maintenance requirements as a result of adaptability, tolerance to poor environments, ability to establish in marginal areas, and a degree of resistance to herbivory from insects and mammals, owing to the toughness and low nutritional value of the leaves (Benett 2010). These factors collectively contributed to reduced input for maintenance, resulting in more economically feasible plantations, which translated into higher profit margins (Pallett and Sale 2004).

In the 1980s, ZAR30 million of pulpwood was exported from South Africa annually, which represented a substantial contribution to the economy as employment opportunities were limited at the time (Rotberg 1980). According to Godsmark and Oberholzer (2017), the forestry industry in South Africa has shown significant growth since then, from a gross value output of ZAR3.266 billion in 2002 to ZAR8.988 billion in 2016. In 2016, *Eucalyptus* species comprised of 42.7% of the total plantation area in South Africa, translating into over half a million hectares of land being cultivated for commercial purposes. This directly created over 60 000 jobs, with almost a further 100 000 indirect jobs for those involved downstream of the timber plantation value chain (Godsmark and Oberholzer 2017).

Methods of Eucalyptus *propagation*

Propagation by seed

Eucalyptus have traditionally been propagated via the collection and sowing of seeds from trees that possess superior growth qualities such as drought and frost tolerance, pest resistance, low lignin content and fast growth rates (Franclet and Boulay 1982; Sharma and Ramamurthy 2000; de Almeida et al. 2015). This propagation strategy has limitations as seeds typically lack true-to-type clonal propagation characteristics due to cross pollination, which results in a loss of the valuable *Eucalyptus* genotype provided by the mother tree (Trueman et al. 2018). This is not a desirable outcome for forestry plantations as the provenance of seed is generally unknown and will result in forests that lack consistency and predictability in growth, form and vigour (Nakhooda and Jain 2016). Furthermore, irregular flowering and seed sets, as well as high embryo abortion rates, are a common feature observed in certain *Eucalyptus* species such as *E. dunii* and *E.*

nitens. These irregularities may result in an inconsistent seed supply, as some seasons will produce a higher number of seeds than others, resulting in an inadequate seed supply in particular seasons, which is problematic for planning of large-scale projects (Ahuja 1993; Hung and Trueman 2011). Lastly, *Eucalyptus* spp. have to reach maturity before the seeds are viable for sowing, which is a time-consuming and laborious process. As such, forestry companies have pursued developing vegetative propagation strategies to preserve valuable genotypes by cloning superior *Eucalyptus* cultivars, which have been reported with varying degrees of success (Nakhooda and Jain 2016).

Vegetative propagation by cuttings

'Totipotency' refers to the capability of an individual, undifferentiated plant cell to differentiate into any of the organs of the plant from which it was isolated (Verdeil et al. 2007). This property is exploited in the vegetative propagation of plants, and is central to the success of commercial forestry programs (Thorpe 1995; Nakhooda and Jain 2016).

Macropropagation is a form of post-embryonic asexual reproduction where a stem cutting is taken from parental material and induced to develop new adventitious roots, in order to form an independent new plant which is genetically identical to the donor or parental plant, i.e. clonally propagated (Rawat et al. 1994). In order to source stem cuttings, *Eucalyptus* plantations are periodically cut at the stem, near ground level, to stimulate re-growth from epicormic buds, in a practice commonly referred to as coppicing (Strong and Zavitkovski 1983). Epicormic buds sprout in response to stress, which can be elicited by manual defoliation or felling of the plant above ground level (Stone and Stone 1943; Meier et al. 2012). Trees can also form these epicormic buds naturally with an increase in light, nutrients and water or when damaged (Ishii and Ford 2001).

The formation of adventitious roots has proven to be a limiting factor in many vegetative propagation programs (Li et al. 2009; Hartmann et al. 2011; Pijut et al. 2012). In many cases, preferred eucalypt genotypes may be excluded from commercial forests because of their inability to reliably form adventitious roots. In addition, even for genotypes that do form adventitious roots easily, an increase in the age of the parent material corresponds

with a decrease in rooting ability (Baccarin et al. 2015). Generally, the more juvenile the parent material, the greater its predisposition to rooting (Brondani et al. 2018). Vegetative rescue practices provide a means to restore juvenility, even for aged parent material. These practices include coppicing or micropropagation, which has shown to increase the propagule amenability to adventitious rooting (Wendling et al. 2010).

Vegetative rescue via coppicing has a potential pitfall referred to as the 'gradient of juvenility', which results in a decrease in juvenility the further away the cuttings are made from the tree base (Hartmann et al. 2011). Therefore, the position from which the propagule is sourced in relation to the tree base is associated with dissimilar rhizogenic responses. Additionally, this rhizogenic response is influenced by a host of exogenous and endogenous factors that include propagule type, humidity, light incidence, physiological and ontogenetic state, tree age as well as hormonal balance (Li et al. 2009; Pijut et al. 2012; de Almeida et al. 2015). Collectively these factors influence the propagule amenability to adventitious rooting, which compromises the predictability and effectiveness of this approach to vegetatively propagate tree species in commercial forests (Hartmann et al. 2011). Therefore, *in vitro* approaches, such as micropropagation, which allow for greater control over the juvenile state of propagules have found favour.

In vitro *propagation*

Tissue culture is a system centred around maintaining highly controlled axenic conditions within culture vessels that allows for the optimum growth of entire plants, tissues or cells (Phillips and Garda 2019). The culture vessel contains a basal medium that provides all the necessary water, vitamins and energy required to rear healthy plants (George et al. 2008 b). Furthermore, the growth conditions for the plants are optimised beyond the culture vessel as these vessels are often incubated in growth chambers to provide optimum illumination and temperature. Plant growth regulators (PGRs) are generally supplemented into the medium at different stages to trigger plant development, depending on the purpose of the culture stage.

Axenic plant cultures are obtained via effective decontamination protocols, and if these conditions are not maintained throughout the life-cycle of the culture, microorganisms such as bacteria, fungi and yeast take advantage of the high nutrient conditions in the culture medium and compete with the explants, resulting in contamination and usually the death of the plant cultures (Watt et al. 2003; Brondani et al. 2011). In order to exclude contaminants from entering the medium, and to maintain axenic cultures, all culture media and equipment need to be autoclaved, and explants are handled using aseptic techniques in a laminar flow hood (Mineo 1990; Ikenganyia et al. 2017).

Micropropagation strategies involve various aseptic techniques, nutrient media and controlled environmental conditions to regenerate entire plants, *in vitro*, from cells, tissues or organs (Debergh and Read 1991; George 2008 b). This strategy is often employed to rapidly multiply numerous plants whilst simultaneously preserving juvenility and desirable genetic traits, which, for commercial eucalypt forests, refers to those cultivars that show dense wood characteristics, shorter rotational periods, cold or drought tolerance, pest resistance and biomass accumulation in the trunk as opposed to the leaves (Nakhooda and Jain 2016). During micropropagation, various organs such as roots, shoots and buds may be induced via controlled supplementation of growth regulators, carbohydrates, minerals, vitamins and temperature (Fehér et al. 2003). Furthermore, the space requirements for micropropagation are far less than those required for macropropagatory strategies (Davey and Anthony 2010). In addition to the aforementioned benefits, micropropagation shows great promise as a strategy to produce transgenic *Eucalyptus* plants in order to study the function and effects of genes for a particular clone or to possibly enhance the genetic potential of the eucalypt (Ahad et al. 2018). As evidenced in this section, micropropagation strategies are highly controlled and therefore provide many solutions to challenges such as the maintenance of juvenility and the subsequent improvement of adventitious rooting ability of superior genotypes for commercial eucalypt forests. In addition, *in vitro*-derived plants, once established into clonal hedges, are reported to offer far greater numbers of shoots for plantation stands than those derived from traditional vegetative propagation practices.

In addition to propagation, tissue culture offers a means to preserve genetically superior *Eucalyptus* germplasm *in vitro* for extended periods of time whilst eliminating the need for large scale land investment, fertilizer, pesticide, labour and water required for silviculture-based germplasm banks (Watt et al. 2000 a; b; Ipekei and Gozukirmizi 2003). Various techniques have been employed in order to preserve valuable *Eucalyptus* genotypes *in vitro*, including synthetic seeds and cryopreservation (Padayachee et al. 2009; Reddy et al. 2012).

Micropropagation of *Eucalyptus* can be achieved via direct and indirect organogenesis (Nakhooda and Jain 2016; Trueman et al. 2018). These strategies have been utilized to establish cultures for common eucalypt forestry species such as *E. grandis* (Watt et al. 1991), *E. camaldulensis* (Girijashankar 2012), *E. citriodora* (Muralidharan and Mascarenhas 1987), *E. polybractea* (Fernando et al. 2016) and *E. globulus* (Nugent et al. 2001), to name a few.

Direct organogenesis is a route that utilises the totipotent nature of plant cells to differentiate vegetative plant tissue directly into adventitious shoots or roots, without any intervening callus stage (Dibax et al. 2010). Indirect organogenesis incorporates the initial formation of callus from vegetative plant tissue, followed by the regeneration of callus into plant organs which may result in somaclonal variation due to possible mutations in the dividing callus (Aggarwal et al. 2010). Somaclonal variation will cause genetic mutations to the germplasm of the *in vitro* cultures, which will negatively impact propagation projects that require uniformity of the germplasm (Leva et al. 2012). *In vitro* plant development via indirect organogenesis has been performed on numerous *Eucalyptus* spp. as reviewed by Trueman et al. (2018). Callus induction during indirect organogenesis is conducted in darkness or in some cases in light, on semi solid medium or in liquid cultures (Bandyopadhyay et al. 1999; Dibax et al. 2010).

The various stages of *in vitro* culture are controlled and characterised by the addition of one or more PGRs, which control all aspects of plant physiological development (Gaspar et al. 1996). Therefore, the foundation to plant tissue culture strategies often revolves around the careful selection and concentrations of the PGRs used.

Role(s) of plant growth regulators

PGRs serve as chemical messengers that collectively co-ordinate the development and growth of plants, from cells to organs (Gaspar et al. 1996). These regulators are classified into several groups that include cytokinins, auxins, abscisic acid, gibberellins, ethylene, brassinosteroids and jasmonates (Fishel 2006; Rademacher 2015), with many more chemicals being reported to possess plant regulatory activities, such as strigolactones (Makhzoum et al. 2015; Ahmad and Anis 2019). Strigolactones are a class of plant hormone that are derived from carotenoids and serve a signalling function, as with other PGRs (Al-Babili and Bouwmeester 2015). These hormones have been known to enhance or inhibit cellular division or elongation dependent upon the tissue or species in question (Hoffmann et al. 2014). Auxins and cytokinins are generally used in combination to elicit their effects, although, the relative concentrations of these PGRs to one another are carefully considered as they may often behave as antagonists (Yang et al. 2017). Cytokinins are responsible for cell division or cytokinesis (Riou-khamlichi et al. 1999). They are known to favour axillary bud growth, and hence cytokinins such as 6-benzylaminopurine (BAP), kinetin and *trans*-zeatin (TZ) are often incorporated into growth media in order to induce shoots, or promote shoot proliferation (Akiyoshi et al. 1984; George et al. 2008 a). In contrast, auxins are responsible for root induction, and also influence several other developmental aspects in plants, such as cell expansion and tropism (Rayle et al. 1992; George et al. 2008 a; Enders and Strader 2015). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are naturally-occurring auxins that are primarily used for the *in vitro* adventitious rooting of *Eucalyptus* cultures (Jones and van Staden 1993; Ho et al. 1998). IBA has been shown to stimulate *in vitro* rooting in many *Eucalyptus* species, some of which include; *E. grandis x urophylla* (Jones and van Staden 1993), *E. benthamii* (Brondani et al. 2012), *E. camaldulensis* (Gupta et al. 1983) *E. saligna* and *E. globulus* (Fett-Neto et al. 2001), and in most cases is favoured over IAA (James 1983). This is because IBA is more stable than IAA, which progressively becomes photo-oxidised to form inactive conjugates within media (Forgaca and Fett-Neto 2005). Other synthetic auxins such as 1-naphthaleneacetic acid (NAA) have also shown prolonged effects *in vitro* when compared with IAA, resulting in a longer lasting effect on *in vitro*

explants, thus potentially increasing root stimulation and producing more roots in certain eucalypts (Li et al. 2009; Pop et al. 2011). The ratios of auxin:cytokinin are carefully selected, depending on the route of organogenesis required. However, unbalanced ratios or excessive concentrations of these PGRs lead to callus formation, which is undesirable in direct organogenesis protocols (Gaspar et al. 1996). Callus consists of an unorganised mass of plant parenchyma cells (Tabata et al. 1973) and, given the relative ease with which calli can be transformed, selected and differentiated (via indirect organogenesis), callus has found use in transgenic studies (Ahad et al. 2018). Often, callus is induced from *Eucalyptus* plant tissues with combinations of PGRs such as cytokinins (BA, BAP and kinetin) and auxins (IAA, NAA and IBA) that are supplemented into the medium, with the auxin concentrations generally exceeding the concentration of cytokinins (Glocke et al. 2005; Hajari et al. 2006; Aggarwal et al. 2010). Furthermore, the formation of adventitious shoots may be stimulated from callus, which are then multiplied in order to produce large stocks of plant material via the use of cytokinins in the medium. Once these shoots have been sufficiently multiplied, they are treated with various auxins to induce adventitious rooting of the shoots (Hajari et al. 2006; Brondani et al. 2012; Girijashankar 2012; Nakhooda et al. 2012). Nevertheless, in most clonal propagation programmes of *Eucalyptus* species, callus formation is problematic, particularly during the rooting stages (Bunn et al. 2015). Often scar callus can form at the base of shoots which displaces or interferes with *in vitro* root formation (Joshi et al. 2003). In the latter stages of culture development, the scar callus may differentiate into roots and serve as a physical barrier, preventing the flow of nutrients and water from the root vasculature to the shoot vasculature (Jones and van Staden 1993; Bunn et al. 2005). Hence auxin:cytokinin ratios need to be carefully considered prior to their supplementation into *Eucalyptus* culture media (Nakhooda et al. 2013). Most reported protocols for the *in vitro* propagation of commercially-important eucalypt clones describe the direct organogenic route.

Shoot culture

Shoot tip cultures, similar to axillary bud cultures, are established via the use of cuttings where the shoot meristematic tissues, such as the axillar and apical buds, remain intact

and are stimulated via PGRs to favour bud outgrowth *in vitro* (Bhatia 2015; Trueman et al. 2018). Shoot tip and axillary bud cultures are essentially very similar and only differ in terms of the initial starting material, where shoot tip cultures are initiated with the apical bud alone and axillary bud cultures are initiated with axillary buds (Vieitez and Vieitez 1980). Often a combination of axillary and apical buds is used to establish *in vitro* cultures of various plant species and many authors have coined "shoot culture" as the terminology to refer to both (Trueman et al. 2018). Shoot cultures are effective at generating a large number of independent explants which are genetically identical to the donor plant; this is beneficial especially when plant material is limited (Le Roux and van Staden 1991; Shanthi et al. 2015). Reportedly, several types of basal medium including Murashige and Skoog salts (MS; Murashige and Skoog 1962), Driver and Kuniyuki walnut medium (DKW; Driver and Kuniyuki 1984), Juan, Antonio, Diva and Silva medium (JADS; Correia et al. 1995), *Eucalyptus dunnii* basal medium (EDM; Oberschelp et al. 2015) and woody plant medium (WPM; Lloyd and McCown 1981) have been effectively utilised for *in vitro* shoot proliferation of *Eucalyptus* (Brondani et al. 2012; Oberschelp and Gonçalves 2016). These basal media are often supplemented with organic additives (myo-inositol, nicotinic acid, pyridoxine-HCl and thiamine-HCl), vitamins (calcium pantothenate and biotin) or mixtures of additives such as de Fossard nutrients (de Fossard et al. 1974; Gomes and Canhoto 2003; Trueman et al. 2018). *Eucalyptus in vitro* shoot proliferation is often conducted in the light, on semi-solid medium containing either Gelrite® or agar (gelling agents) and 15-20 g ℓ^{-1} sucrose in addition to the aforementioned basal media (Jones and van Staden 1993; Pinto et al. 2007). Outgrowth of the buds present on *Eucalyptus* explants is promoted via the supplementation of various cytokinins, such as kinetin and BAP into the proliferation medium (Jones and van Staden 1993; Brondani et al. 2011). Reported protocols for eucalypt culture are often clone- or species- specific, and hence modifications of protocols are often necessary to ensure the success of eucalypt micropropagation for each clone (Nakhooda et al. 2011; 2012; 2013; Brondani et al. 2012). Ideally a single protocol designed for a broader spectrum of clones would be preferable for industrial applications as this will reduce the cost and time required to develop a new protocol when switching between *Eucalyptus* clones for specific applications.

The rooting capabilities among different *Eucalyptus* clones have also proven to be clone-specific, as some clones may be more responsive to the auxins supplied *in vitro* than others (Mankessi et al. 2009), contributing to the difficulty in developing a universal rooting protocol. For example, some eucalypt clones develop excessive basal callus in the presence of a given concentration of IBA, which may be insufficient to induce adventitious rooting in another clone given the same concentration of the hormone (Nakhooda et al. 2014). This excessive callus hinders the uptake of water and nutrients, and thus compromises *Eucalyptus* plant survival during the acclimatization phase (Bunn et al. 2015). Hence, PGRs have to be carefully selected throughout the *in vitro* developmental period, and varied depending on the stage of the culture period.

Stages of Eucalyptus *micropropagation*

Selection and preparation of source material

Many forestry programmes grow trees in clonal hedges which are used as the starting material for large scale cultivation projects (Watt et al. 1995). Since woody plant species, including *Eucalyptus*, are host to thousands of microbial organisms which include fungal and bacterial endophytes (Faeth and Fagan 2002), parental plants require treatment and maintenance prior to establishment *in vitro* for micropropagation (Watt et al. 2003). In order to reduce microbial contamination, the parental material is generally treated with fungicides such as Benlate® (1 g ℓ^{-1}) and Kumulus DF® (3 g ℓ^{-1}) or with systemic fungicides such as Previcur® (1 g ℓ^{-1}) to eliminate endophytic fungi (Watt et al. 2003; Brondani et al. 2011; van der Wolf et al. 2012). Studies by Ting et al. (2014), have illustrated that treatment of parental material with the antibiotic streptomycin (0.01 mg mℓ^{-1}) was effective at eliminating bacterial contaminants from propagules.

Healthy cuttings are vital for successful proliferation of explants during micropropagation and desiccation of cuttings prior to *in vitro* establishment hinders micropropagation efficiency. To avoid desiccation of cuttings, parent material should be harvested when temperatures are low, such as in the morning, after rain or in a humid environment of a greenhouse (Sein and Mitlohner 2011). The onset of desiccation can be further prolonged

through immediate placement of explants into culture or, if necessary, storage in humid conditions preceding their transfer into the growth medium (Naidu and Jones 2009). The ability of shoot cultures to form roots *in vitro* has been shown to correlate with the distance at which cuttings are harvested from the shoot apex (Bryant and Trueman 2015). This is due to the fact that the further the distance from the shoot apex, the higher the level of sclerification, where protoplasts of mainly parenchyma cells die off and the plant cell wall thickens through the accumulation of lignin (Machado et al. 2005). These sclerenchymatous cells are referred to as sclereids and are usually dead cells (Marjamaa et al. 2007). Higher levels of sclerification have been observed in *Eucalyptus* species, such as *E. nitens*, *E. tetrodonta* and *E. pilularis*, the further cuttings are made from the shoot apex (Bryant and Trueman 2015). This higher degree of sclerification has been shown to reduce the rootability of *C. torelliana* × *C. citriodora* clones *in vitro* (Wendling et al. 2015).

Decontamination

Prior to transfer *in vitro*, decontamination is crucial and is frequently the limiting factor to the success of micropropagation protocols (Trueman et al. 2018). Regular sterilisation of equipment used during harvesting should reduce the spread of contaminants from cutting to cutting. Subsequent to felling, the cuttings can be further treated with 70% ethanol, Previcur® or Benlate® to minimize microbial proliferation. The cuttings should be immersed in ascorbic acid (10 g ℓ^{-1}) or Previcur® and Benlate® (1 g ℓ^{-1}) prior to transportation to initiate the treatment process (Watt et al. 2003; Brondani et al. 2012).

During decontamination *Eucalyptus* cuttings are trimmed down to a third of the original size in order to reduce the surface area to be decontaminated and to reduce the probability of contaminants (Jones and van Staden 1993). These cuttings are treated systematically with various solutions which consist of boric acid, Benlate®, Previcur®, gentamycin, Tween-20®, sodium hypochlorite and mercuric chloride to eradicate all forms of microbial contaminants that include fungi, bacteria and yeast (Nakhooda et al. 2011). Sterilants such as the chlorine-based sodium hypochlorite (NaOCl), is recommended over

mercuric chloride ($HgCl_2$), regardless of the fact that $HgCl_2$ has proven to be a stringent component in decontamination solutions (Sharma and Ramamurthy 2000; Watt et al. 2006). This is because $HgCl_2$ has exhibited prolonged environmental persistence and high mammalian toxicity (Park and Zheng 2012). Tween-20® is added to the decontamination solutions as a surfactant or wetting agent to improve the surface contact between the sterilant and plant material, which is especially necessary when working with eucalypts, as the leaf and stem regions tend to contain hair-like trichomes (Trueman et al. 2018). Subsequent to decontamination, the explants are rinsed with sterile water before being transferred into media to remove the harmful residue chemicals carried over from the decontamination stage, which may be detrimental to the newly transferred explants (Le Roux and van Staden 1991; Jones and van Staden 1993; Brondani et al. 2012).

In vitro *establishment and multiplication*

The purpose of the multiplication stage of micropropagation is to establish as well as to multiply the shoot explants *in vitro* post decontamination. Typically, in eucalypts, this is achieved via stimulation of axillary and apical buds to form new shoots in a medium containing cytokinins, usually BAP, TZ, thidiazuron (TDZ) and/or kinetin. This initial stage may also be referred to in the literature as bud induction (Huetteman and Preece 1993; Brondani et al. 2011; Nakhooda et al. 2013). The cytokinins are intended to promote shoot multiplication, cell division and axillary bud formation (George et al. 2008 a). The concentration of cytokinins added to the media needs careful consideration as an excess will result in cytokinin persistence in the explants, which will have an antagonistic effect on the future rootability of *Eucalyptus* clones (Nakhooda et al. 2012). Persistence can occur when the cytokinins used for shoot proliferation are either stable against degradation *in vitro* or used in excess, resulting in carry-over into the rooting stages, where they may behave antagonistically to the rhizogenic action of auxins (Nakhooda et al. 2013). To reduce cytokinin persistence, lower concentrations or less stable cytokinins, such as TZ, that are rapidly degraded may sometimes be recommended (Mok and Mok

2001; Nakhooda et al. 2012). The multiplication phase also serves to acclimatise the explants from field-derived parent material to the highly-controlled *in vitro* environment. The success of the multiplication phase may be quantified by the number of explants produced per cutting with an initial number of nodes (Brondani et al. 2012).

Elongation

Elongation stages are often incorporated into the micropropagation strategy to condition the shoot material for rooting and, as the name suggests, may sometimes be necessary for elongating the shoots, as longer shoots tend to root more effectively in certain *Eucalyptus* species (Nakhooda and Jain 2016). Increased rootability of *Eucalyptus* explants has been reported with an elongation stage that incorporates low concentrations of cytokinins and auxins in the culture medium (Nakhooda et al. 2011). Studies have reported elongation in medium devoid of PGRs in *E. grandis x E. urophylla* and *E. tereticornis x E. grandis* hybrids, followed by improved rooting performance *in vitro* (*Joshi* et al. 2003). Incorporation of 0.1 mg ℓ^{-1} IBA into the elongation media has reportedly induced a high level of shoot elongation and vigour in *E. benthanii*, when compared to media with gibberellic acid (GA) and/or BAP, which resulted in shoots that were fragile, consisted of fewer leaves and which were unable to root during the rooting stage (Brondani et al. 2011). The stability of the various auxin analogues used during elongation influences the subsequent rooting stages as some auxins are more stable and will persist within the explants into the rooting stages, leading to an auxin bias or priming effect which generally favours adventitious root formation. However, a surplus of auxin in the rooting medium, due to persistence in the explants, may result in basal callus formation (Pacurar et al. 2014).

In vitro *rooting*

Rooting of *Eucalyptus* clones has been reported with varying degrees of success and is seen as a pivotal factor which dictates the success or failure of a micropropagation

programme (de Klerk et al. 1999). The rooting of *Eucalyptus* explants requires optimisation of four factors which all influence the rooting potential of the *in vitro* explants. These are 1) the genotype of the *Eucalyptus* clone to be rooted; 2) temperature and light exposure; 3) ratio and concentrations of exogenous PGRs; and 4) macro- and micro-nutrient requirements (Nakhooda and Jain 2016). Rooting can be optimised via careful manipulation of these factors to determine which are antagonistic and which are complementary in achieving the best rooting possible for a particular *Eucalyptus* clone.

Prior to transfer into rooting medium, the *Eucalyptus* shoots are often trimmed at the base of the stem to reveal the vasculature, this wounding is significant to the formation of adventitious roots (de Klerk et al. 1999). As previously mentioned, roots which develop from plant organs/tissues are termed adventitious and are divided into two forms, namely latent or wound induced adventitious roots (Carlson 1938). Latent roots tend to remain dormant and only emerge once conditions are favourable for root development (Lovell and White 1986). The second type of adventitious root is the one that forms the basis for the majority of vegetative propagation programs, which is induced once an incision or wound is made at the base of a stem or leaf (Carlson 1938; Steffens and Rasmussen 2016).

PGRs work together to co-ordinate growth physiology via intracellular communication and explant development is extremely sensitive to shifts in concentrations of these regulators. The emphasis is therefore generally placed upon selection of the most suitable PGRs for a given suite of clones (Gaspar et al. 1996). The auxin IBA has proven to produce the greatest root number, density and percentage, in *E. saligna* and *E. globulus*, compared with to NAA and IAA (Forgaca and Fett-Neto 2005). This response may be related to the increased relative stability of IBA, which is five-times less readily photo-oxidised than IAA (Nissen and Sutter 1990). Other authors have suggested that this may be due to the slow conversion of IBA to IAA, serving as a steady release source of an easily metabolized IAA (Forgaca and Fett-Neto 2005). Due to the stable nature of IBA, this PGR requires careful consideration as higher dosages in species such as *E. sideroxylon* has been shown to induce callus as IBA remains present in the explants and media for extended periods of time, the resulting callus can have an antagonistic effect to root production

(Cheng et al. 1992). However, others have reported rooting without callus formation with the use of IBA in eucalypts such as *E. camaldulensis*, further illustrating how responses differ based on the *Eucalyptus* species (Girijashankar 2012). An auxin-free rooting medium has also been shown to allow root formation in some *Eucalyptus* spp., which is seen in cases where stable auxins have been used in the prior multiplication and elongation stages that persist in the explants and carry over into the rooting stages, thus inducing root formation (Nakhooda et al. 2011). On the other hand, the use of stable cytokinins such as kinetin, in the stages preceding rooting has been shown to negatively affect root development by acting antagonistically to the auxins provided during rooting. Kinetin has shown to reduce root number and percentage due to its prolonged persistence in the plants (Nakhooda et al. 2013). Consequently, a hormone-free elongation medium prior to rooting may be considered to flush out persisting cytokinins. However, this observation is not limited to cytokinins and may apply to stable auxins that could persist in explants leading to a super-optimal concentration of auxins during the rooting stage.

Macro- and micro- nutrients also play a part in the development of healthy adventitious roots. They serve as charge carriers, play a part in osmoregulation of plant cells and activate numerous enzymatic reactions (Schwambach et al. 2005). According to Schwambach et al. (2005), the addition of mineral nutrients such as nitrate (18 mM), Zn (30 µM and 60 µM) and Ca (15 mM) have been shown to positively influence root density and length in *E. globulus*. Furthermore, in that study, calcium was used at much higher concentrations when compared with zinc and nitrate, which is due to the fact that calcium is a macro-element that is required in larger amounts compared to the latter nutrients which are micro- and required in trace amounts. This study also found a reduction in root length as well as root browning when ammonium was used as a nitrogen source (Schwambach et al. 2005). No significant effects were noted when phosphorus and potassium were supplemented into the medium, but an excess or lack of these macro-nutrients resulted in a reduction in root number and length (Schwambach et al. 2005). Research conducted on *E. grandis x E. nitens* hybrids has shown a reduction in callus formation and an increase in rooting performance when supplementing media with

calcium and magnesium, as well as when the concentration of MS was reduced from a full strength to quarter strength in the medium (Mokotedi et al. 2000).

The addition of activated charcoal to the rooting medium during eucalypt micropropagation has also been documented, based on reports of activated charcoal acting as an agent to reduce phenolic oxidation, absorb inhibitory compounds, reduce irradiance and alter the pH of the medium around the base of the stem to enhance explant growth (Trueman et al. 2018). Studies by Jones and van Staden (1993) illustrated the improved effects of activated charcoal on rooting of *E. gandis x urophylla* hybrids, where enhanced rooting was observed when 1 % (wv^{-1}) activated charcoal was incorporated into the rooting medium. PGRs are generally omitted from media containing activated charcoal as the adsorption of auxins and cytokinins from the medium renders these phytohormones inactive (Pan and van Staden 1998). Alternatively, other anti-browning agents which can be added to the medium include ascorbate and polyvinylpyrrolidone (Brondani et al. 2011; Shanthi et al. 2015; Trueman et al. 2018).

Acclimatization

Following the *in vitro* stages of micropropagation, the resulting plants should contain the necessary systems such as root, shoot and leaves in order to be self-sufficient in *ex vitro* conditions. During acclimatization of *Eucalyptus*, the plant is introduced gradually to the outside environment to experience conditions such as wind, rain, sun and cold at an increasing intensity (Brondani et al. 2011; Girijashankar 2012). The increasing exposure to the external environment is generally controlled via a greenhouse with high levels of mist irrigation (Jones and van Staden 1993). The mist provides a humid environment similar to that experienced *in vitro*, to prevent desiccation of the plants which may not have fully developed cuticles and stomata; these only begin to develop as the plants become more exposed to direct sunlight and dryer conditions (Beckett et al. 2013). The vascular systems of the plants also require further development during acclimatization stages as the roots have not been exposed to the soil environment preceding acclimatization. Hence, providing the plants with an easily accessible source of

carbohydrates and vitamins, such as MS, is important before allowing the plants to become fully independent (Padayachee et al. 2008). This can be achieved via supplementation of sugars or MS into the watering systems provided to the plants, and gradually reducing the content of these supplements in the water so that the plants become self-sufficient at sequestering nutrients from the soil medium in the field. To protect the plants from pathogens, the soil and pots can be decontaminated before explant transfer. The plants should also be sprayed weekly with fungicides and insecticides until they have grown sufficiently and are more resistant to pathogenic infection (Le Roux and van Staden 1991). Heating of the soil or substrate has been shown to improve root growth during the acclimatization process (Nakhooda and Jain 2016). Presently, forestry companies tend to establish acclimatised *in vitro* plants into clonal hedges, to serve as parent material from which cuttings are taken for clonal forestry stands (Nakhooda and Jain 2016).

Eucalyptus *gene expression patterns offer clues to* in vitro *performance*

It has been observed that the published protocols for *Eucalyptus* micropropagation are clone specific, requiring optimisation for every genotype selected (Mankessi et al. 2009; Brondani et al. 2012). However, the underlying mechanisms behind these observed clonal variations are poorly understood, particularly during adventitious root formation (de Almeida et al. 2010). The correct supply of PGRs is central during micropropagation, as these influence and regulate plant physiology and development, and as such are the starting point for the optimisation of *in vitro* protocols (Phillips and Garda 2019). The influence of PGRs on *Eucalyptus* clones may be attributed to the particular clone's ability to transport, perceive or synthesise these hormones (de Almeida et al. 2010). As already discussed, auxins serve a crucial role during adventitious root development, and the clonal responses may rely heavily on the endogenous concentration and transport rate of auxin (Fogaça CM and Fett-Neto 2005). Auxin biosynthesis is localised at the shoot apex, carried out partly by proteins such as YUC3 and TAA1, and is the primary source of endogenous auxin (de Almeida et al. 2015). These auxins are distributed basipetally

through the plant by active transport, which is conducted via the combination of influx (AUX1) and efflux (PIN) carriers (Muday and de Long 2001; René and Scheres 2008). The distributed auxins are perceived by the plant tissues via auxin binding protein (ABP1), an auxin receptor, believed to be associated with primary responses of cell growth (Tromas et al. 2010). Collectively, the expression of these proteins may affect the rooting ability as well as growth of *Eucalyptus* clones. However, limited information regarding the role(s) of auxin on the development of root meristems in woody plant species is available (Li et al. 2009; de Almeida et al. 2015). The changes in gene expression associated with the aforementioned proteins can be evaluated via reverse transcription of extracted mRNA, followed by quantitative polymerase chain reaction (qPCR). This technique offers a high degree of specificity and sensitivity for the targeted genes and allows for measurements of gene expression in multiple samples simultaneously (Bustin 2002; Gachon et al. 2004). Certain factors require consideration in order to reproducibly and accurately assess gene expression. These include the quantity of starting material, the quality of RNA isolation and subsequent cDNA synthesis, inhibitors present in samples and primer design (Ginzinger 2002). Therefore, RT-qPCR involves the incorporation of relevant normalization strategies which are crucial for procuring reliable and accurate information regarding gene expression levels in a sample (Huggett et al. 2005). Endogenous reference genes are commonly incorporated as a normalization strategy for RT-qPCR, improving the accuracy of quantification (Li et al. 2017). Appropriate reference or housekeeping genes are presumably constitutively expressed genes, such that these genes are expressed at a consistent level in all of the tissues and organs of the study organisms, as well as at different stages of development (Rebouças et al. 2013). Appropriate normalizers are generally involved in cellular processes, which includes primary metabolism or structure maintenance (Czechowski et al. 2005). Numerous reference genes have been identified for the study of *Eucalyptus* gene expression, which have been outlined and tested by de Almeida et al. (2010). These normalizers have been utilised to study the effects of exogenously supplied auxin, namely IAA, on the gene expression levels in *E. globulus* and *E. grandis* (de Almeida et al. 2015). Thus, the use of RT-qPCR may potentially prove to be a useful tool to study the patterns of expression in

Eucalyptus clones to potentially identify differences in gene expression that may elucidate the variable clonal growth responses seen *in vitro*.

Study aims and objectives

The micropropagation of *Eucalyptus* is often unpredictable in terms of clonal responses to various *in vitro* conditions (Mankessi et al. 2009; Brondani et al. 2012). The majority of publicised protocols have typically been empirical and as such, are highly clone specific. This study proposes a novel strategy of testing *in vitro* protocol, via the incorporation of an intermediate maintenance medium, to incubate explant material and use these explants as source material for subsequent *in vitro* growth experiments where required. This will enable each respective growth stage to be studied independently. Therefore, the present investigation aimed to develop a unique approach to identify the responses of novel *Eucalyptus* clones to various PGRs, with the ultimate goal of seamlessly establishing these plants to *in vitro* conditions and optimising protocol where required. This study represents a rapid means of identifying the clonal response to various PGRs *in vitro*, and determining the optimal treatment for each respective stage of *in vitro* propagation via the process of elimination to omit treatments that elicit poor clonal growth responses. This process may save the forestry industry time and resources as the optimal treatments are initially identified and retained prior to testing the effects of each stage of micropropagation in succession. This strategy was tested on three commercially-important *Eucalyptus grandis x nitens* hybrids, received from a forestry company in South Africa. The selected *Eucalyptus* clones, according to the forestry company, exhibited differential responses to *in vitro* propagation and a high degree of recalcitrance to adventitious rooting, and as such, rarely survived the acclimatization process.

In order to understand the underlying mechanisms responsible for the observed *in vitro* clonal variation as well as rooting recalcitrance amongst these *Eucalyptus* hybrids, analysis of the how the key PGR, namely IAA, is transported, perceived and synthesised was conducted via RT-qPCR (real time-quantitative polymerase chain reaction) in an attempt to identify a correlation between the level of gene expression within the clones

and the observed phenotypic responses *in vitro*. IAA was selected based on its relevance to adventitious rooting and during *in vitro* elongation (Pop et al. 2011). Differences in the perception, biosynthesis or transport of IAA by each clone may consequently be a factor that explains why the clones respond differently to *in vitro* propagation protocols (de Almeida *et al.* 2015).

To this end, the *Eucalyptus* hybrids were cultured in three independent *in vitro* propagation stages to determine the clonal responses to exogenous PGR supply. The three commonly implemented stages included multiplication to proliferate the shoot material, elongation to prepare the shoot material for *in vitro* rooting and lastly rooting. The optimal treatments for each respective micropropagation stage, as well as the clonal responses to the treatments were identified. Subsequent to the *in vitro* stages, acclimatization was performed to determine the ability of each clone to survive in the field following the optimal *in vitro* rooting protocol currently designed. Lastly RT-qPCR was conducted, using the auxin related genes *PIN1*, *AUX1*, *YUC3* and *ABP1* to determine whether the responses observed during *in vitro* propagation may be related to a difference in the ability of each *Eucalyptus* clone to perceive, transport or synthesise exogenous IAA.

CHAPTER II

Materials and methods

Parent material obtained for culture initiation[1]

Cuttings from three different clones (named clones1-3), of the hybrid *Eucalyptus grandis x nitens* were obtained from the KwaZulu-Natal region in 2017, for *in vitro* establishment. Each clone was approximately 6 months old and displayed disparate responses to *in vitro* organogenesis. These cuttings were transported in Ziploc® bags to Stellenbosch University (Western Cape) via a courier service, which took approximately 4 d. Systematic treatment to decontaminate the *Eucalyptus* cuttings and ultimately establish axenic cultures was conducted using Benlate®, boric acid, Tween20, mercuric chloride and calcium hypochlorite[1].

In vitro *conditions and culture medium preparation*

All the chemicals required for the purposes of this study were sourced from Promega (Madison, WI, USA) and Sigma Aldrich Fluka (St. Louis, MO, USA), unless stated otherwise. The pH of all *in vitro* media in the current study was adjusted to 5.8, using 0.1 M KOH. Following the adjustment of the pH, 2.8 g ℓ^{-1} Gelrite® was added to the culture medium, prior to autoclaving. All of the media and apparatus necessary for *in vitro* tissue culture were autoclaved at 121°C (≈ 1.0 kgf cm^{-2}) for 20 min. Subsequent to autoclaving, all media was allowed to cool to 60°C prior to the addition of phytohormones and vitamins, which were filter-sterilized with a Millex-GP® filter (0.22 µm sterile membrane). Culture media preparation and handling, post autoclave, was conducted in a laminar flow

[1] Decontamination protocols were tested and reported in 2017 by Rafael Keret during BSc (Hons) research.

chamber using aseptic techniques. Incubation of the *Eucalyptus* cultures was conducted in a controlled growth room at 23°C (± 2°C), under cool white fluorescent lighting (Osram L 58V/740) with a 16 h photoperiod (50 µmol photons $m^{-2}s^{-1}$ of luminosity) followed by an 8 h dark period.

In vitro *initiation, maintenance, multiplication and elongation media preparation*

The *in vitro* shoots were initially cultured in polycarbonate magenta vessels (575.113 cm^3) containing 80-100 mℓ of medium Mt5 (full strength MS with vitamins, 0.1 mg $ℓ^{-1}$ calcium pantothenate, 0.1 mg $ℓ^{-1}$ biotin, 0.1 mg $ℓ^{-1}$ IAA, 0.5 mg $ℓ^{-1}$ BAP, 0.1 mg $ℓ^{-1}$ kinetin, 20 g $ℓ^{-1}$ sucrose and 2.8 g $ℓ^{-1}$ Gelrite®)(Table 2.1) in order to acclimate the shoots to *in vitro* conditions and to proliferate the plant material to generate sufficient stock plants for experimentation. Subsequently, once sufficient stock plants had been generated, the shoots were transferred into maintenance medium (full strength MS with vitamins, 0.1 mg $ℓ^{-1}$ calcium pantothenate, 0.1 mg $ℓ^{-1}$ biotin, 0.05 mg $ℓ^{-1}$ IAA, 0.025 mg $ℓ^{-1}$ BAP, 20 g $ℓ^{-1}$ sucrose and 2.8 g $ℓ^{-1}$ Gelrite®) with 12 shoots per magenta vessel and sub-cultured every 4 weeks, onto fresh maintenance medium. The maintenance medium served as a means to incubate the stock plant material of all three *Eucalyptus* clones, whilst using minimal PGRs as possible to prevent carry-over of hormones that may have adverse effects on subsequent *in vitro* experiments that require PGRs in specific controlled amounts, thus eliminating bias. The maintenance medium was developed and presently implemented to quantify each respective *in vitro* growth stage independently, i.e. multiplication, elongation and rooting in isolation, to develop a strategy for rapidly identifying individual clonal responses to a given suite of PGRs. Therefore, explants sourced for the multiplication, elongation and rooting experiments originated from shoots established in maintenance medium, so that the growth responses elicited from each respective growth stage can be directly related to a particular treatment, with minimal influences of carry over PGRs.

In vitro *multiplication and elongation experimental outline*

The multiplication and elongation experiments were independently initiated from shoots sourced from maintenance medium. These were trimmed to contain five buds (four axillary and one apical) per shoot and to a size of between 0.7 cm and 1.6 cm. Trimming of the shoots included cutting the base of the stem to reveal the shoot vasculature. The longitudinal measurement of the shoot length was taken from the excision site to the apical bud using a Vernier calliper (Figure 2.1 a). Five multiplication media (Mt1-Mt5) and four elongation media (Et1-Et4) were tested, varying in type and concentration of the PGRs (Table 2.1). Both the multiplication and elongation experiments were performed in a randomized complete block design, testing different media with each of the three *Eucalyptus* clones. Each replicate consisted of twelve shoots per magenta vessel (Figure 2.1 b), placed into 100 mℓ of medium at the excision site. Each experiment contained 3 replicates per treatment (n=36). Following a 28 d culture period, the shoot length and bud number was determined and the relative change in these variables from the start of the culture period was calculated (Figure 2.1 c; d).

In vitro *rooting*

Ten rooting media (Rt1-Rt10) were tested, varying in the PGR and supplement type as well as concentration (Table 2.1). The organic biostimulant BC204, which consists of a citrus extract with various organic acids, and the synthetic strigolactone GR24, were also supplemented into various rooting media along with IAA (Table 2.1). Additionally, rooting treatments Rt1 and Rt2 contained a higher concentration of MS and sucrose compared to the other rooting treatments. The *Eucalyptus* shoots, sourced from maintenance medium, were cut at the base of the stem to expose the shoot vasculature. Single shoots of longer than 1.2 cm were transferred into flat-bottom culture tubes (49.09 cm^3) containing 15 mℓ of rooting medium (Figure 2.1 e). Following a 28 d growth period, the plants were carefully extracted from the rooting medium. The length of the roots was measured using digital Vernier callipers, from the location of root emergence to the root

tip. Furthermore, the root number was determined by counting the number of individual adventitious roots formed per shoot, roots that developed from leaves were excluded from the root number count. The rooting experiment was performed in a randomized complete block design. All rooting experiments comprised of 36 shoots, with the top three rooting experiments, in terms of rooting percentage, repeated in triplicate (n=108).

Acclimatization of in vitro *rooted clones*

Rooted shoots from rooting medium 9 (Rt9, Table 2.1) were used for the acclimatization experiment. These plantlets were carefully removed from the culture medium, ensuring minimal damage to the roots. Excess Gelrite® was washed off the root surface using sterile autoclaved water to reduce the occurrence of bacterial and fungal contaminants being introduced to the roots. The plantlets were transferred into soil pots (735.8 cm^3) which were inserted into trays to elevate the pots from the water at the base of the acclimatization chamber (Figure 2.1 f). The soil mixture comprised of autoclaved palm peat, sand and vermiculite in a 3:1:1 ratio. The soil was saturated with water containing ½ strength MS and the trays were placed into a clear 50 ℓ PVC container (Figure 2.1 f). The container was modified to contain six lid plugs, each measuring 25 mm in diameter. The plantlets were incubated in this growth chamber for 3 d, after which one lid plug was removed at random every third day, until there were no plugs remaining after 21 d. The plants were allowed to grow under these conditions for a further 3 d, with the lid being completely removed after 24 d. Half strength MS was sprayed onto the plants each time a lid plug was removed, i.e. every third day, until day 21, after which the plants were watered without MS supplementation. The survival of the plantlets was documented every third day for a period of 30 d. This experiment was repeated once with 8 biological repeats per clone.

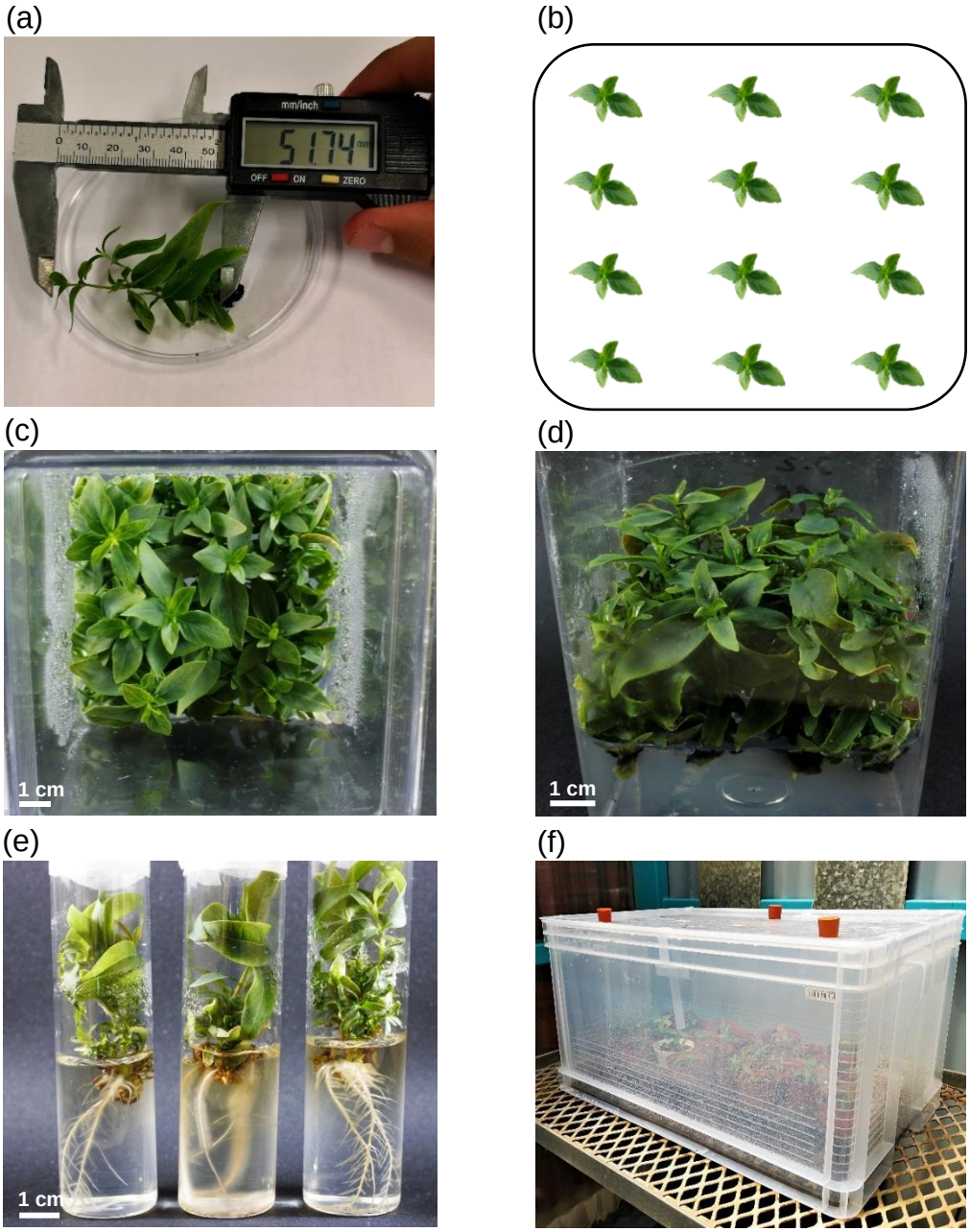

Figure 2.1 (a) Digital Vernier calipers utilized to measure shoot and root length; **(b)** Typical distribution of the *Eucalyptus* explants in the culture vessels during the multiplication and elongation stages; **(c)** Depiction of the typical culture layout in multiplication media, following a 28 d growth period, the size bar represents 1 cm; **(d)** Typical culture layout in elongation media, following a 28 d growth period, the size bar represents 1 cm; **(e)** *Eucalyptus* culture layout in rooting media, following a 28 d growth period, the size bar represents 1 cm; **(f)** Chamber for acclimatization of *Eucalyptus* plantlets following *in vitro* propagation, photographed on day 12.

Table 2.1 The respective concentrations of the various PGRs and supplements incorporated into the multiplication (Mt1-Mt5), elongation (Et1-Et4) and rooting (Rt1-Rt10) media [2].

Culture medium composition	Mt1	Mt2	Mt3	Mt4	Mt5	Et1	Et2	Et3	Et4	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7	Rt8	Rt9	Rt10
PGR (mg ℓ$^{-1}$)																			
IAA	0.1	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5	0.1	0.5	0.1	0.5	–	–	–	0.5	0.5	–
IBA	–	–	–	–	–	–	–	–	–	–	–	–	–	0.1	0.5	–	–	–	–
NAA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.1	–	–	–
GR24[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.029	–
BAP	0.05	0.25	0.1	–	0.5	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–
Kinetin	0.5	0.25	0.1	–	0.1	–	0.05	–	–	–	–	–	–	–	–	–	–	–	–
Meta-topolin	–	–	–	0.5	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–
Supplement (g ℓ$^{-1}$)																			
MS	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Vitamin B5 (mg ℓ$^{-1}$)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Biotin (mg ℓ$^{-1}$)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose	20	20	20	20	20	20	20	20	20	20	20	15	15	15	15	15	15	15	15
BC2O4[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.1	–	–
Activated charcoal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10

[1] Synthetic strigolactone. [2] Organic biostimulant synthesized from citrus extracts and organic acids.

[2] Plant growth regulator and supplement concentrations are represented in molar in Appendix 1.

Expression analysis of genes involved in auxin biosynthesis, transport and perception

Total RNA was extracted 48 h after the shoots were placed into the rooting medium 4 (Rt 4), to determine the effects of exogenous auxin (IAA) supplementation on gene expression. IAA was selected as this auxin was prevalent in all the *in vitro* growth stages, thus the effects of this auxin on all the stages of growth may be inferred based upon the ability of each clone to effectively utilise this PGR. The RNA extraction was repeated separately for all three *Eucalyptus* clones. The genes chosen to be analysed in the present study were selected based on their involvement in auxin transport, biosynthesis and perception (Table 2.2), as reported by de Almeida et al. (2015).

Total RNA extraction and DNase treatment

Three shoots per clone were flash frozen and ground in liquid nitrogen to a fine powder using a pre-cooled ceramic mortar and pestle. Total RNA was extracted in a Maxwell® 16 LEV instrument from 50-100 mg homogenised samples using the Maxwell® 16 Cell LEV Total RNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). The RNA samples were treated with a DNase I kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol, to remove gDNA contamination. The integrity of the RNA samples was analysed by subjecting 300 ng of RNA, with 2x RNA loading dye (Thermo Fisher Scientific, USA), to gel electrophoresis on a 1% (w/v) TBE agarose gel, run at 100V.

Reverse transcription of the isolated RNA into complementary DNA (cDNA)

The DNase-treated RNA extracts were subjected to first strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol and normalised for each sample by using 720 ng of RNA extract. The negative control, for each clone, was conducted via the omission of reverse

transcriptase from the cDNA synthesis reaction. The synthesized cDNA was stored for future use at -80°C.

Testing of the synthesized cDNA using GoTaq® PCR

To verify the reverse transcription of RNA into cDNA and to test the specificity of the primer sets, a PCR was performed using the GoTaq® DNA Polymerase kit (Promega, USA) according to the cycling protocol recommended for optimal GoTaq® activity. The PCR cycling stages were conducted in a T100™ Thermal Cycler (Bio-Rad, USA) using the published primer sets by de Almeida et al. (2015), described in Table 2.2. The PCR reactions were incubated at 95°C for 5 min for initial denaturation, followed by 40 cycles of 95°C for 15 s (Denaturation), 60 °C for 15 s (Annealing), 72 °C for 15 s (Extension) and a final single cycle of 72 °C for 10 min (Final extension). The reagents used in the PCR reactions are outlined in Table 2.3. The PCR products were analysed for amplification and specificity via gel electrophoresis on a 2% (w/v) TBE agarose gel, run at 100V.

Table 2.2 Primer sequences obtained from de Almeida et al. (2015) to study the expression of genes involved in auxin transport, biosynthesis and perception in *Eucalyptus* clones.

Primer	Sequence (5'-3')	Amplicon size	Function	Gene name
***PIN1* Fp**	ACCTCATGGTCCAGATCGTC	143 bp	Auxin efflux carrier	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
***PIN1* Rp**	ACCTCGGATGGAGATGATGG			
***AUX1* Fp**	CAGCTTCAAGAACCACGTCA	107 bp	Auxin influx carrier	Auxin transporter protein 1
***AUX1* Rp**	CGAAGAGGAGGAAAGTGCAG			
***YUC3* Fp**	AGCTTCACCTTCCAAAGCAA	123 bp	Auxin biosynthesis	YUCCA 1
***YUC3* Rp**	TGTCAAAGTGCCTGGCATAG			
***ABP1* Fp**	TTGCAGCCACTTTCTGACTG	160 bp	Auxin perception	Auxin binding protein 1
***ABP1* Rp**	GATCAAACATCGGGGTATGC			
[1]*H2B* Fp	GAAGAAGCGGGTGAAGAAGA	145 bp	Structural constituent	Histone H2B
[1]*H2B* Rp	GGCGAGTTTCTCGAAGATGT			
[1]*TUA* Fp	ACCGGTTGATCTCTCAGGTG	103 bp	Structural constituent	Alpha-tubulin
[1]*TUA* Rp	TAAGGGACCAGGTTGGTCTG			

[1]Reference genes used in this study.

Table 2.3 Various reagents and the respective volume of each used for the GoTaq® PCR reaction of the genes involved in auxin transport, biosynthesis and perception.

PCR reagent	Volume (µℓ)
5X Green GoTaq® Reaction Buffer	5
10 mM dNTP Mix	0.75
10 mM Forward Primer	0.75
10 mM Reverse Primer	0.75
(5 U/µℓ) GoTaq® DNA Polymerase	0.1
cDNA	1
Nuclease-Free Water	16.65
Final volume	25

Two-step RT-qPCR of the genes involved in auxin transport, biosynthesis and perception

A dilution series (10^{-1} to 10^{-4}) of the previously synthesised cDNA from each *Eucalyptus* clone was created. Subsequently, each cDNA dilution was subject to reverse transcriptase quantitative PCR (RT-qPCR), via the addition of 1 µℓ of cDNA and 0.8 µℓ of primer (Table 2.2), to the PowerUp™SYBR™ Green Master Mix (Thermo Fisher Scientific, USA), as per manufacturer's recommendations. The reactions were performed in a 0.1 mℓ, MicroAmp™, optical 96-well clear reaction plate (Applied Biosystems, USA) using a Quantstudio™ 3 Real-Time PCR system (Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. The incubations for the RT-qPCR reactions were as follows: 95°C for 10 min to activate the Dual-Lock *Taq* DNA polymerase, followed by 40 cycles of 95°C for 15 s (Denaturation) and 60°C for 1 min (Annealing and Extension). Subsequent to the final RT-qPCR cycle, a heat dissociation curve was generated (Melt curve stage), from 60°C to 95°C to determine the primer specificity for the RT-qPCR reactions.

The results obtained from the dilution series were extracted from the Quantstudio™ design and analysis software (Thermo Fisher Scientific, USA) to generate a standard

curve for each gene in Excel (Microsoft, USA). The primer amplification efficiencies for every gene were determined from the linear slope of the standard curve, primers that failed to demonstrate a primer efficiency between 95-105%, were considered inefficient omitted from further statistical analysis (Yun et al. 2006).

To determine the relative gene expression, the aforementioned RT-qPCR experiment was repeated with the 10-fold cDNA dilution for all three of the *Eucalyptus* clones. The gene expression data from the Quantstudio™ design and analysis software (Thermo Fisher Scientific, USA), were analysed via the comparative Ct method (Schmittgen and Livak 2008) and the calculation of relative expression was conducted with each reference gene separately. The normalisation procedure revealed similar relative expression results for both reference genes. Consequently, H2B was selected for normalisation to determine the fold expression.

Statistical analysis

Multiplication, elongation and rooting data analysis

Prism 7 software (GraphPad Software, USA) was used to analyse, transform and visualize the data sets for the multiplication, elongation and rooting experiments. The multiplication, elongation and rooting data were assessed for normality using the Shapiro Wilk test. Non-Gaussian-distribution was observed in certain data sets. Consequently, data transformations were performed, using a square root transformation, resulting in normally distributed data sets for the multiplication and elongation data. These data sets were analysed via two-way ANOVA with Tukey's HSD to distinguish statistically significant ($p \leq 0.05$) differences that existed between the means of the treatments as well as the clones. The transformations failed to normalise the rooting data sets and thus a non-parametric approach was implemented to analyse the rooting data. The rooting data was analysed using the Kruskal-Wallis *H* test and Friedman test with a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to distinguish statistically significant differences for mean root length and number between the treatments and clones

respectively, using a 95% confidence interval ($p \leq 0.05$). The rooting percentages were analysed via the chi-squared test of independence, for each of the clones.

RT-qPCR data analysis

The delta-CT (ΔCt) values were used to determine the significant differences in gene expression as the use of this raw data eliminates any bias that may arise from further downstream data manipulations (Brown et al. 2018). Prism 7 software (GraphPad Software, USA) was used to analyse ΔCt and visualize $2^{-\Delta\Delta CT}$ generated from the RT-qPCR data. The ΔCt data were assessed for normality using the Shapiro Wilk test, with Gaussian distribution observed in all the data sets. Consequently, the data was analysed using one-way ANOVA, to compare the relative gene expression between the three *Eucalyptus* clones for a given gene, i.e. relative expression of AUX1 between each *Eucalyptus* clone (Figure 3.13 a). Additionally, the T-test was used to compare the relative expression between two genes for a given *Eucalyptus* clone, i.e. relative expression of *AUX1* to *PIN1* for each clone (Figure 3.13 b). Tukey's HSD *post hoc* test was used for both of these parametric tests to distinguish statistically significant ($p \leq 0.05$) differences that existed between the mean gene expression value.

CHAPTER III

Results

The results section represents the findings obtained from the implementation of the multiplication, elongation, rooting and gene expression study experiments. These findings portray the clonal responses toward the treatments with PGRs and supplements, based upon the type and concentration. Furthermore, the optimal treatment(s) have been identified for each respective growth stage in this chapter. The numerical values of the experiments are tabulated in the Appendix section of this dissertation.

Effects of multiplication treatments on bud proliferation

The multiplication treatments tested in this study had an elevated ratio of cytokinin to auxin, with the cytokinin content typically twice that of auxin (Table 2.1). This ratio of PGR theoretically favours bud proliferation (George et al. 2008 a), which was observed for each of the clones for all the treatments tested.

Clone 1 displayed an increase in bud number in response to all of the multiplication media treatments, following a growth period of 28 d (Figure 3.2 a). Shoots cultured in Mt1-Mt3 produced significantly fewer buds than shoots proliferated in Mt4 and Mt5, although significant differences in bud proliferation were also present amongst the first three treatments relative to each other, with Mt1 resulting in fewer buds than Mt2 and Mt3 (Figure 3.2 a). The optimal multiplication treatments for clone 1, consisting of 0.5 mg ℓ^{-1} *meta*-topolin (Mt4) and BAP (Mt5), produced a mean increase in bud number, following 28 d of growth, of 59.1 (± 2.9) and 51.7 (± 2.42) buds per shoot respectively, with no significant differences observed between these treatments. Evidently the optimal bud proliferation for this clone was achieved via an increase in BAP concentration, from 0.05 mg ℓ^{-1}, until an optimal concentration of 0.5 mg ℓ^{-1} was reached. Conversely, a decrease

in kinetin, from 0.5 mg ℓ^{-1} to a concentration of 0.1 mg ℓ^{-1} resulted in a significant increase in mean bud number, from 17.3 (± 1.4) in Mt1, to 31.1 (± 1.4) buds per shoot upon treatment in Mt3. Additionally, the presence of *meta*-topolin at 0.5 mg ℓ^{-1} as the only PGR in the growth medium (Mt4), elicited a significantly higher level of bud proliferation in comparison to shoots treated with higher concentrations of kinetin or low concentrations of BAP, used in Mt1-Mt3 (Figure 3.1 a).

Clone 2 exhibited a significant increase in bud number in response to all of the multiplication treatments tested in this study, following a 28 d growth period (Figure 3.2 a). However, shoots treated with an elevated kinetin concentration of 0.5 mg ℓ^{-1} (Mt1), displayed a significantly lower mean increase in bud number (36.3 ± 1.3) compared to those treated with lower concentrations of kinetin or BAP and *meta*-topolon (Mt2-Mt5). Treatments, Mt2-Mt5, displayed a mean proliferation of 65.1 (± 2.3), 65.1 (± 2.4), 62.3 (± 2.7) and 68 (± 2.7), buds per shoot respectively. No significant differences in bud proliferation were observed amongst the shoots treated with Mt2-Mt5, with respect to each other. As for clone 1, a decrease in kinetin concentration from 0.5 mg ℓ^{-1} to 0.1 - 0.25 mg ℓ^{-1} enhanced bud proliferation for clone 2. By way of contrast, clone 2 produced the highest change in bud number in response to various concentrations of BAP and to *meta*-topolin (Figure 3.1 b), typically exceeding a proliferation of 65 buds per shoot in response to both of these PGRs, over a 28 d growth period.

Similarly, shoots of clone 3 displayed bud proliferation in response to all multiplication treatments tested (Figure 3.2 a). The highest bud proliferation for shoots of clone 3 was obtained when they were cultured on 0.1 mg ℓ^{-1} of BAP and kinetin (Mt3), *meta*-topolin (Mt4) and 0.5 mg ℓ^{-1} of BAP (Mt5), displaying an average increase of 50.5 (± 2.4), 58.1 (± 3) and 54.9 (± 2.2), buds per shoot respectively after 28 d, with no significant differences between these treatments. Like clones 1-2, bud proliferation of clone 3 displayed an inversely proportional relationship to a high concentration of kinetin (0.5 mg ℓ^{-1}). Thus, at a reduced kinetin concentration of 0.1 mg ℓ^{-1} in the multiplication medium, bud proliferation was significantly enhanced. In contrast to clones 1 and 2, however, the response of clone 3 to BAP treatment was variable and no clear trends in growth were observed in response to a progressive decrease or increase in BAP concentration. The incorporation of *meta*-

topolin at 0.5 mg ℓ^{-1} (Mt4) into the growth medium, significantly enhanced bud proliferation compared to shoots treated on medium containing 0.05 mg ℓ^{-1} of BAP with 0.5 mg ℓ^{-1} of kinetin (Mt1) or treatments with 0.25 mg ℓ^{-1} of BAP and kinetin (Mt2) (Figure 3.1 c).

In response to the five multiplication treatments implemented in the present investigation, clone 2 displayed the highest overall level of bud proliferation in comparison to the other two clones. This indicates that clone 2 was the most amenable to *in vitro* stimulation by the PGRs tested, while clone 1 appeared to be the most recalcitrant of the three clones (Figure 3.2 a) to shoot multiplication. The bud proliferation in response to the treatments tested indicated an intermediate response by clone 3, when compared to the other clones. In terms of treatment consistency, treatments incorporating high concentrations of BAP and *meta*-topolin at 0.5 mg ℓ^{-1}, induced the highest levels of bud proliferation amongst all the clones tested, as opposed to high kinetin treatment at 0.5 mg ℓ^{-1}. Therefore, the optimal multiplication treatments for shoot proliferation in the tested clones include a high concentration of BAP and *meta*-topolin in the growth medium.

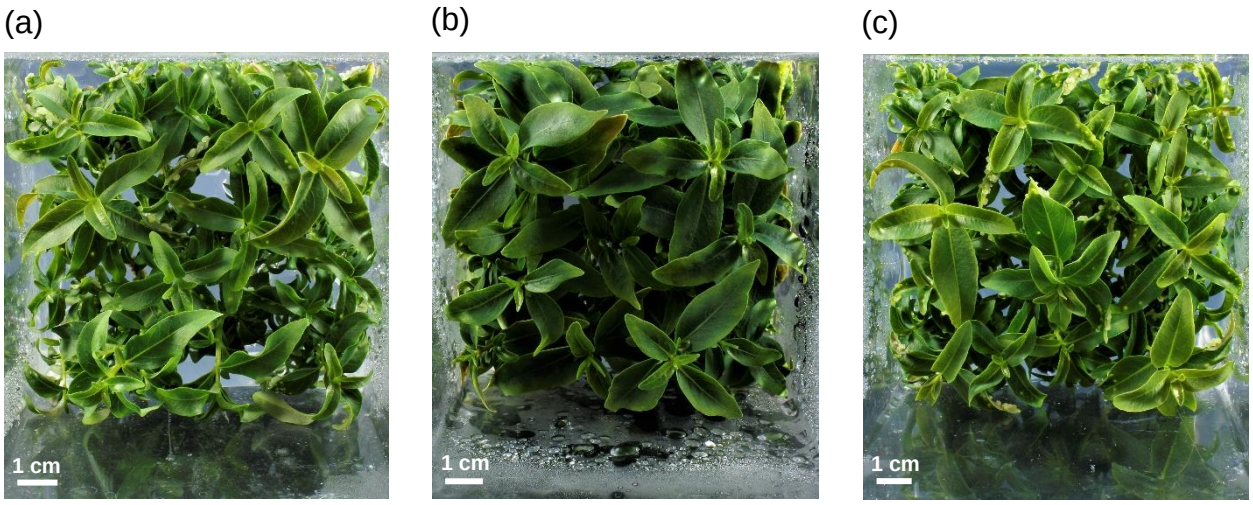

Figure 3.1 (a) Multiplied shoots of clone 1; **(b)** clone 2 and; **(c)** clone 3 upon exogenous supplementation of 0.5 mg ℓ^{-1} of *meta*-topolin (Mt4) into the growth medium. The figures represent multiplied shoots following a 4-week growth period, with size bars depicting 1 cm.

Effects of multiplication treatments on shoot size

Whilst the multiplication medium was intended for shoot proliferation, variations in shoot size was also observed in many of the treatments, with clonal response also evident in this regard. The tallest shoots for clone 1 were obtained upon supplementation of *meta*-topolin at 0.5 mg ℓ^{-1} (Mt4) into the growth medium, yielding a mean shoot growth of 1.2 cm (± 0.08), which was significantly higher than those shoots that were treated with IAA, IBA, kinetin or BAP, which displayed an increase in shoot length of 0.4 cm (± 0.04; Mt1), 0.4 cm (± 0.04; Mt2), 0.9 cm (± 0.08; Mt3) and 0.7 cm (± 0.04; Mt5) over the course of 28 culture days. Shoot length was enhanced in response to a gradual decrease of kinetin from 0.5 mg ℓ^{-1} (Mt1) to 0.1 mg ℓ^{-1} (Mt3 and Mt5), no obvious trends in shoot length were observed in relation to an increase or decrease in BAP concentration.

The growth seen in shoots of clone 2 placed in multiplication medium typically exceeded 1.2 cm in response to all of the treatments tested over 28 d. No significant differences, in terms of stem growth, were observed between the shoots of clone 2 cultured in treatments Mt1, Mt3 and Mt4, which obtained an average shoot growth of 1.8 cm (± 0.09), 1.5 cm (± 0.09) and 1.8 cm (± 0.08) respectively. Shoot length was significantly greater medium containing *meta*-topolin (Mt4) as opposed to that containing 0.25 mg ℓ^{-1} of BAP and kinetin (Mt 2) or 0.5 mg ℓ^{-1} of BAP (Mt5), which displayed an average growth of 1.3 cm (± 0.09) and 1.2 cm (± 0.09), respectively (Figure 3.2 b). The tallest shoots were obtained when the BAP concentration was decreased from 0.5 mg ℓ^{-1} (Mt5) to 0.05 mg ℓ^{-1} (Mt1) and in the presence of *meta*-topolin (0.5 mg ℓ^{-1}).

Typically shoots placed into multiplication medium exhibited growth exceeding 0.7 cm for clone 3 in response to all of the treatments tested over 28 d. Shoots treated with 0.25 mg ℓ^{-1} of BAP and kinetin (Mt2) produced significantly shorter shoots (0.74 cm ± 0.05) as opposed to those treated with 0.05 mg ℓ^{-1} of BAP and 0.5 mg ℓ^{-1} of kinetin (1.1 cm ± 0.06; Mt1) or *meta*-topolin (1.2 cm ± 0.07; Mt4). The remainder of the treatments displayed no significant differences from each other, in terms of shoot length for clone 3. No significant trends were observed in relation to BAP and kinetin concentrations supplemented into the medium.

During multiplication, the responses by the clones to increase in shoot length shared a similar trend to that of bud proliferation. Those results indicated that clone 2 was the most responsive to *in vitro* stimulation by PGRs, producing, on average, longer shoots than the other clones. Similarly, to bud proliferation, clone 3 showed an intermediate response, while clone 1 displayed the shortest shoots (Figure 3.2 b) of the tested clones. Conversely to bud proliferation, the lengthening of the shoots displayed no significant correlation with respect to BAP and kinetin concentrations. However, all clones responded positively to the supplementation of *meta*-topolin at 0.5 mg ℓ^{-1} into the growth medium when compared to the other PGRs tested, and this was consequently the multiplication treatment that produced the tallest shoots.

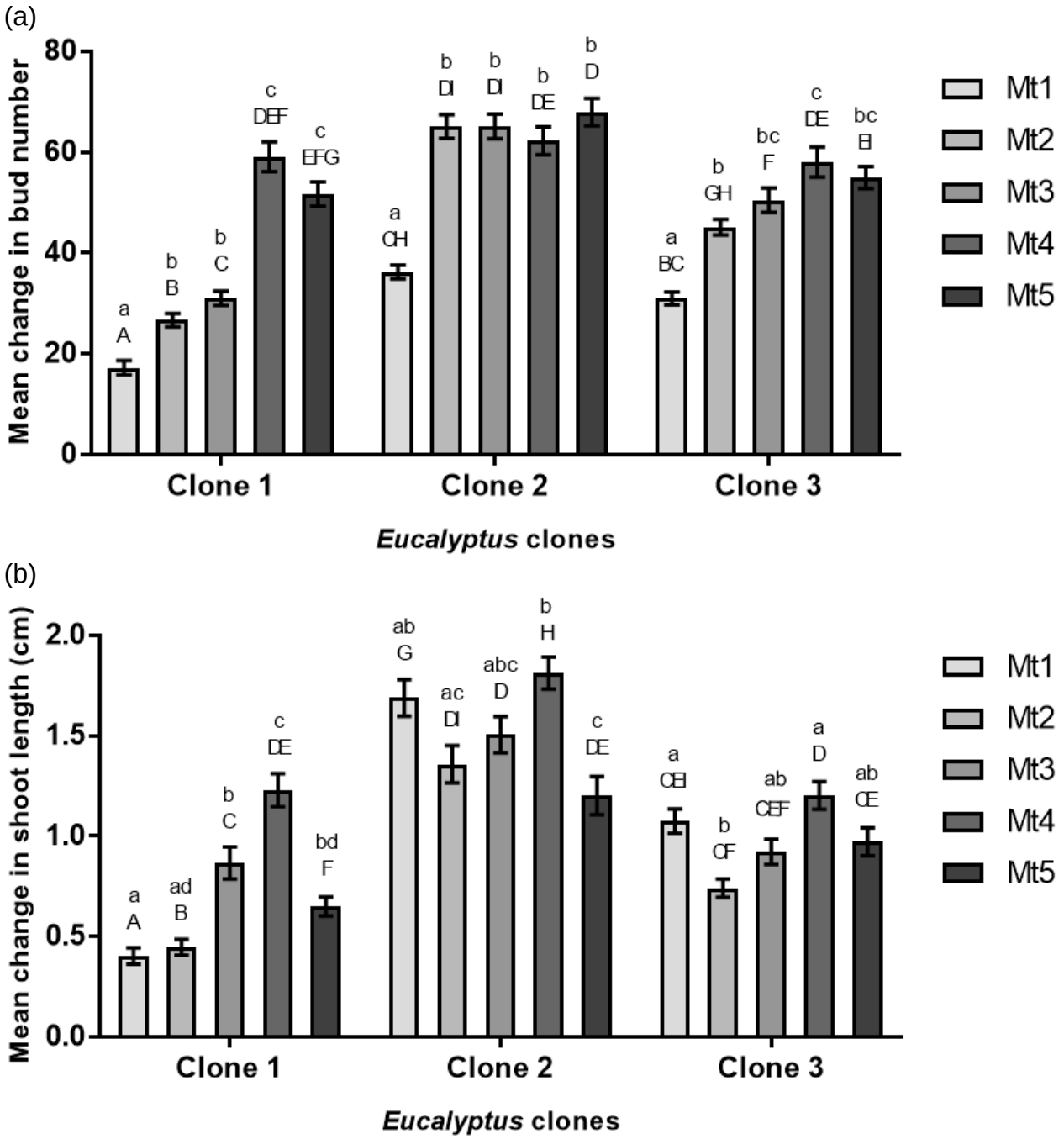

Figure 3.2 The mean change in **(a)** bud number and; **(b)** shoot length for three *Eucalyptus* clones, subject to five multiplication treatments (Mt1-Mt5). The data were analysed using square-root transformed values. The error bars represent the standard error of the mean, lower-case letters denote significant differences between the treatments and upper-case letters indicate significant differences between the clones, analysed via Tukey's HSD with a 95% confidence interval ($p \leq 0.05$). The graphs display the untransformed data values.

Clonal responses to various elongation media treatments

The elongation treatments tested in this study comprised of the auxin, IAA at 0.5 mg ℓ^{-1}, in conjunction with a lower concentration of the cytokinins kinetin (Et2), BAP (Et3) and *meta*-topolin (Et4) at 0.05 mg ℓ^{-1} each. No cytokinins were included in Et1 medium, which contained only IAA.

The highest shoot elongation for clone 1 was observed when shoots were cultured with BAP (Et3) and *meta*-topolin (Et4), yielding an average shoot elongation of 2.1 cm (± 0.06) and 2.6 cm (± 0.07) respectively, which was significantly higher than the elongation obtained with no cytokinins (Et1) (0.88 cm ± 0.09) or kinetin (Et2) (0.49 cm ± 0.06). The shortest shoots for clone 1 were induced on medium containing 0.05 mg ℓ^{-1} of kinetin, over a 28 d growth period (Figure 3.4). This result reflects those obtained during multiplication, where kinetin negatively impacts explant growth for clone 1 (Figure 3.2 a; b). The presence of BAP (Et3) or *meta*-topolin (Et4, Figure 3.3 a) in combination with IAA in the medium resulted in a significant increase in shoot elongation compared to Et1, which comprised of IAA alone (Figure 3.4).

For clone 2, shoot elongation in response to kinetin (Et2) and *meta*-topolin (Et4) was 2.6 cm (± 0.14) and 2.9 cm (± 0.09) respectively, over the culture period. Those treatments resulted in significantly longer shoots than those treated with IAA alone (Et1) or BAP (Et3), which displayed elongated shoot lengths of 1.4 cm (± 0.13) and 2.2 cm (± 0.1) respectively, over the same period. The addition of *meta*-topolin in combination with IAA into the growth medium (Figure 3.3 b) resulted in a significantly lengthier shoots compared to BAP or kinetin in the growth medium. The omission of cytokinins from the elongation medium (Et1) resulted in a significantly reduced shoot elongation for clone 2, compared to all the other media that contained cytokinins.

The highest shoot elongation for clone 3 was obtained in the presence of *meta*-topolin (0.05 mg ℓ^{-1}) and IAA (0.5 mg ℓ^{-1}) in Et4, with an average change in shoot length of 3 cm (± 0.09) over the culture period (Figure 3.3 c). This result was followed by shoots grown on media with BAP (1.8 cm ± 0.09), then IAA alone (1 cm ± 0.06), and lastly on kinetin

(0.5 cm ± 0.05). As with the elongation results for clone 1, shoot elongation for clone 3 was significantly limited by the inclusion of kinetin in the elongation medium.

During elongation, the observed clonal responses towards the four elongation treatments presently tested indicated that clone 2 was most effectively elongated compared to the other tested clones. This indicated that, as observed during multiplication, clone 2 appeared to be the most responsive clone to the tested PGRs *in vitro*. Clones 1 and 3 elongated relatively equally in response to the treatments tested. The omission of cytokinins from the growth medium in Et1 resulted in significantly reduced elongation amongst the clones, in comparison to the inclusion of BAP (Et3) and meta-topolin (Et4). In combination with IAA (0.5 mg ℓ^{-1}), the cytokinin *meta*-topolin (0.05 mg ℓ^{-1}) most consistently induced the highest degree of shoot elongation in the majority of the clones presently tested, producing the tallest shoots.

Additional observations revealed that the *Eucalyptus* explants were well preserved on the maintenance, multiplication and elongation media, rarely displaying necrosis, chlorosis or vitrification following a growth period of 28 d. Furthermore, the maintenance medium effectively preserved the explant material *in vitro*, whilst using minimal PGR concentrations. The explants sourced from maintenance medium were transitioned successfully into all of the subsequent growth stages, where required. The formation of callus around the base of the stem, was frequently present on all of the clones during *in vitro* growth, and did not appear to adversely affect growth, given that the subculture frequency was every 28 d. Furthermore, despite the fact that certain multiplication treatments resulted in shoot lengthening, the elongation step still produced significantly taller shoots in comparison to multiplication.

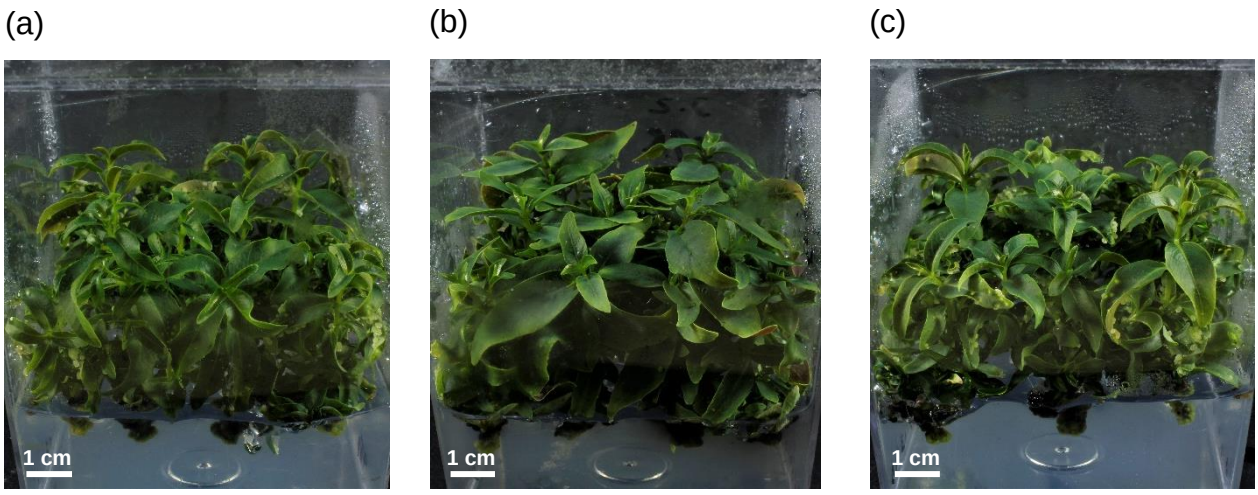

Figure 3.3 (a) Elongated shoots of clone 1; **(b)** clone 2 and; **(c)** clone 3 upon exogenous supplementation of 0.05 mg ℓ^{-1} of *meta*-topolin (Mt4) into the growth medium. The figures represent elongated shoots following a 4-week growth period, with size bars depicting 1 cm.

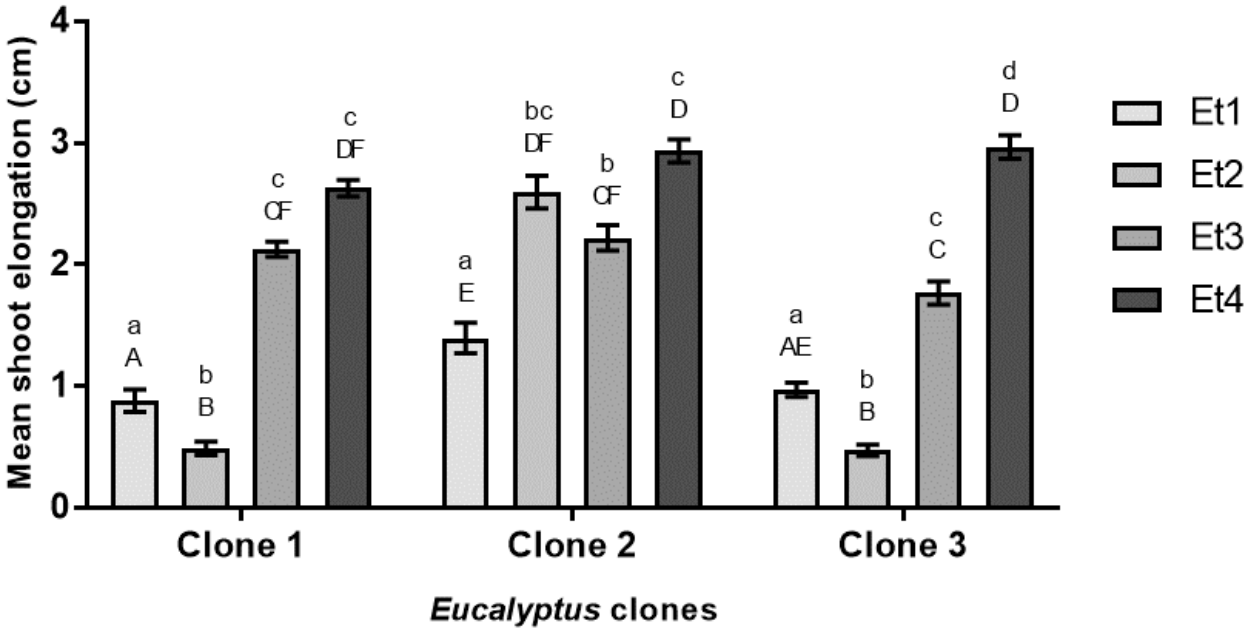

Figure 3.4 Mean shoot elongation for three *Eucalyptus* clones, subject to four elongation treatments (Et1-Et4). The data were analysed using square-root transformed values. The error bars represent the standard error of the mean, the lower-case letters denote whether significant differences exist amongst the treatments and the upper-case letters indicate significant differences amongst the clones, analysed via Tukey's HSD with a 95% confidence interval ($p \leq 0.05$). The graph displays the untransformed data values.

Clonal responses to various rooting media treatments

Effects of rooting treatments on root length, number and percentage rooting

The effects of various auxin types and concentrations on the formation of adventitious roots were tested on the three *Eucalyptus* clones presently under investigation (Table 2.1). Ten different rooting media were tested for each of the *Eucalyptus* clones. Factors such as root length, number and percentage were considered in order to identify the top three rooting treatments for the clones presently tested (Figure 3.5; 3.6; Table 3.1). The chi squared test of independence revealed that the rooting observations or percentage for each *Eucalyptus* clone in relation to the treatments tested were dependent (i.e. rooting

percentage was significantly influenced by the treatment). The association between each treatment tested in relation to adventitious roots formed per shoot (i.e. rooting percentage) were significant (p<0.0001), for clone 1, $X^2(9, N = 36) = 50.27$, clone 2, $X^2(9, N = 36) = 68.14$ and clone 3, $X^2(9, N = 36) = 57.87$.

During the rooting stage, each of the *Eucalyptus* clones displayed disparate rooting responses to the various treatments tested, following a culture period of 28 d. The initial rooting treatments incorporated 0.1 mg ℓ^{-1} (Rt1) and 0.5 mg ℓ^{-1} (Rt2) of IAA in combination with 20 g ℓ^{-1} of sucrose and full-strength MS. These treatments elicited an extremely poor rooting response for all three tested *Eucalyptus* clones, with rooting percentages ranging from 5.56 % - 11.1 % (Table 3.1). Subsequently, a significantly enhanced rooting response was observed in each clone with a reduction of sucrose to 15 g ℓ^{-1} and MS to ¼ strength, in combination with 0.5 mg ℓ^{-1} IAA (Rt4). Although Rt3, containing 0.1 mg ℓ^{-1} IAA, also incorporated a reduced sucrose and MS content, this treatment rarely induced a significantly improved rooting response in terms of root length (Figure 3.5) and number (Figure 3.6), compared to Rt1 and Rt2. However, each of the *Eucalyptus* clones displayed an enhanced rooting percentage with Rt3 (27.8 %, clone 1; 22.2 %, clone 2; 25 %, clone 3) as opposed to Rt1 (8.33 % for all clones) and Rt2 (11.1 %, clone 1; 11.1 %, clone 2; 5.56 %, clone 3). The reduction in sucrose from 20 g ℓ^{-1} to 15 g ℓ^{-1} and full-strength MS to ¼ strength elicited a greater rooting response for all of the *Eucalyptus* clones tested. Consequently, this combination of sucrose and MS was incorporated for the remainder of the treatments. The *Eucalyptus* clones cultured with Rt4 did not produce a significantly higher root length and number compared to Rt3 (Figure 3.5; 3.6), rather an improvement was observed in the rooting percentage, where Rt4 elicited enhanced rooting percentages of 36.1 % (clone 1), 33.3 % (clone 2) and 38.9 % (clone 3). These rooting percentages indicated that adventitious rooting is enhanced with an increase in IAA, as the occurrence of adventitious roots were more prevalent at a concentration of 0.5 mg ℓ^{-1} (Rt4) compared to 0.1 mg ℓ^{-1} (Rt3) of IAA.

Treating the *Eucalyptus* clones with IBA displayed no distinct association between the concentration used and the rooting performance, as each clone displayed a disparate rooting response at a given concentration of IBA. The shoots of clone 1 displayed a

significant enhancement in root length and number in response to a decrease in IBA concentration, from 0.5 mg ℓ^{-1} (Rt6) to 0.1 mg ℓ^{-1} (Rt5), with an improvement in rooting from 13.9 % to 41.7 %, respectively. Similarly, clone 2 displayed a significant increase in root length and percent rooting from 52.8 % (Rt6) to 61.1 % (Rt5) upon a reduction in IBA concentration. However, the number of roots produced by clone 2, did not display a significant difference at both tested IBA concentrations. Although the rooting percentage was enhanced with lower IBA concentrations for clones 1 and 2, the roots formed at the higher IBA concentration appeared thicker and contained a greater abundance of root hairs for all the clones (Figure 3.7 d; e). In contrast to clones 1 and 2, clone 3 displayed significant enhancement in root length and number in response to an increase in IBA concentration, from 0.1 mg ℓ^{-1} (Rt5) to 0.5 mg ℓ^{-1} (Rt6), with an improvement in rooting percentage from 19.4 % to 58.3 % respectively. All of the *Eucalyptus* clones treated with IBA typically rooted more effectively when compared to treatments with IAA (Figure 3.5; 3.6). Whilst IBA and IAA did not significantly influence the root number and length for clone 1, IBA was found to significantly enhance rooting percentage in this clone, compared to IAA. Conversely, treatment of clones 2 and 3 with IBA improved the rooting response for every aspect tested.

The incorporation of BC204 into the growth medium, with 0.5 mg ℓ^{-1} of IAA, did not significantly enhance the rooting response in comparison to IAA alone (Rt4), for all of the *Eucalyptus* clones tested. Although rhizogenesis was not significantly influenced in clone 1 by BC204 in the growth medium, the rooting percentages in clones 2 and 3 were drastically reduced to 13.9 % when BC204 was included in the medium. Similarly, the inclusion of GR24 at 0.029 mg ℓ^{-1} together with 0.5 mg ℓ^{-1} IAA (Rt9) did not significantly enhance the root length and number in any of the *Eucalyptus* clones presently tested. However, clone 2 displayed an increased rooting percentage in response to GR24 from 33.3 % (Rt4) to 44.4 % (Rt9), indicating that supplementation of IAA with GR24 does improve rooting for clone 2. However, despite not having a significant impact on rooting, GR24 seemingly delayed senescence and improved plant vigour in the all of the explants (Figure 3.7 a; b; c), in comparison to the other treatments tested, as necrosis and chlorosis appeared to have been far less prevalent in the cultures with this combination

of PGRs. Furthermore, the shoots grown on Rt9 formed relatively taller and healthier shoots than those grown on the other rooting media that were tested (Figure 3.9 a-i). This was observed in all three of the *Eucalyptus* clones tested.

Lastly, the treatments that induced the poorest rooting response in the *Eucalyptus* clones were 0.1 mg ℓ^{-1} of NAA (Rt7) and 10 g ℓ^{-1} of activated charcoal (Rt10). Upon exogenous supply of NAA no roots were formed for clones 1 and 3, while this treatment resulted in the lowest rooting percentage of 5.56 % for clone 2 compared to the other rooting treatments tested (Table 3.1). No roots were obtained for clone 1 upon supplementation of activated charcoal into the rooting medium, while for clones 2 and 3 this treatment did not give significant improvement over any of the other treatments tested.

The present investigation has revealed that improvement of *in vitro* adventitious root formation was accomplished by a decrease in sucrose from 20-15 g ℓ^{-1} and MS from full to ¼ strength in the rooting medium. The percentage of adventitious roots that developed was increased when the concentration of IAA was increased from 0.1 mg ℓ^{-1} to 0.5 mg ℓ^{-1}, for all of the *Eucalyptus* clones under investigation. Decreasing the IBA concentration from 0.5 mg ℓ^{-1} to 0.1 mg ℓ^{-1} typically displayed a positive impact on adventitious root length, number and percentage for *Eucalyptus* clones 1 and 2. Conversely, clone 3 displayed a significantly improved response to adventitious root length and number in association with an increase in IBA concentration. Furthermore, IBA at 0.5 mg ℓ^{-1} resulted in an abundance of root hairs for all clones tested (Figure 3.7 e). Although no statistical significance was observed with respect to adventitious root length and number, when 0.5 mg ℓ^{-1} of IAA was supplemented with 0.029 mg ℓ^{-1} GR24, a synthetic strigolactone (Rt9), this treatment appeared to delay explant senescence compared with the other treatments tested in this study, GR24 thus appears to be a beneficial additive for rooting experiments.

Table 3.1 Rooting percentages obtained for clones 1, 2 and 3 grown on ten different rooting media (Rt1-Rt10). The emergence of adventitious roots was determined to be dependent on the treatment type, for all the *Eucalyptus* clones tested, by the Chi-squared test of independence.

	% Rooting per treatment									
Clone	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7	Rt8	Rt9	Rt10
1	8.33	11.1	27.8	36.1	41.7	13.9	0	38.9	30.6	0
2	8.33	11.1	22.2	33.3	61.1	52.8	5.56	13.9	44.4	11.1
3	8.33	5.56	25.0	38.9	19.4	58.3	0	13.9	30.6	16.7

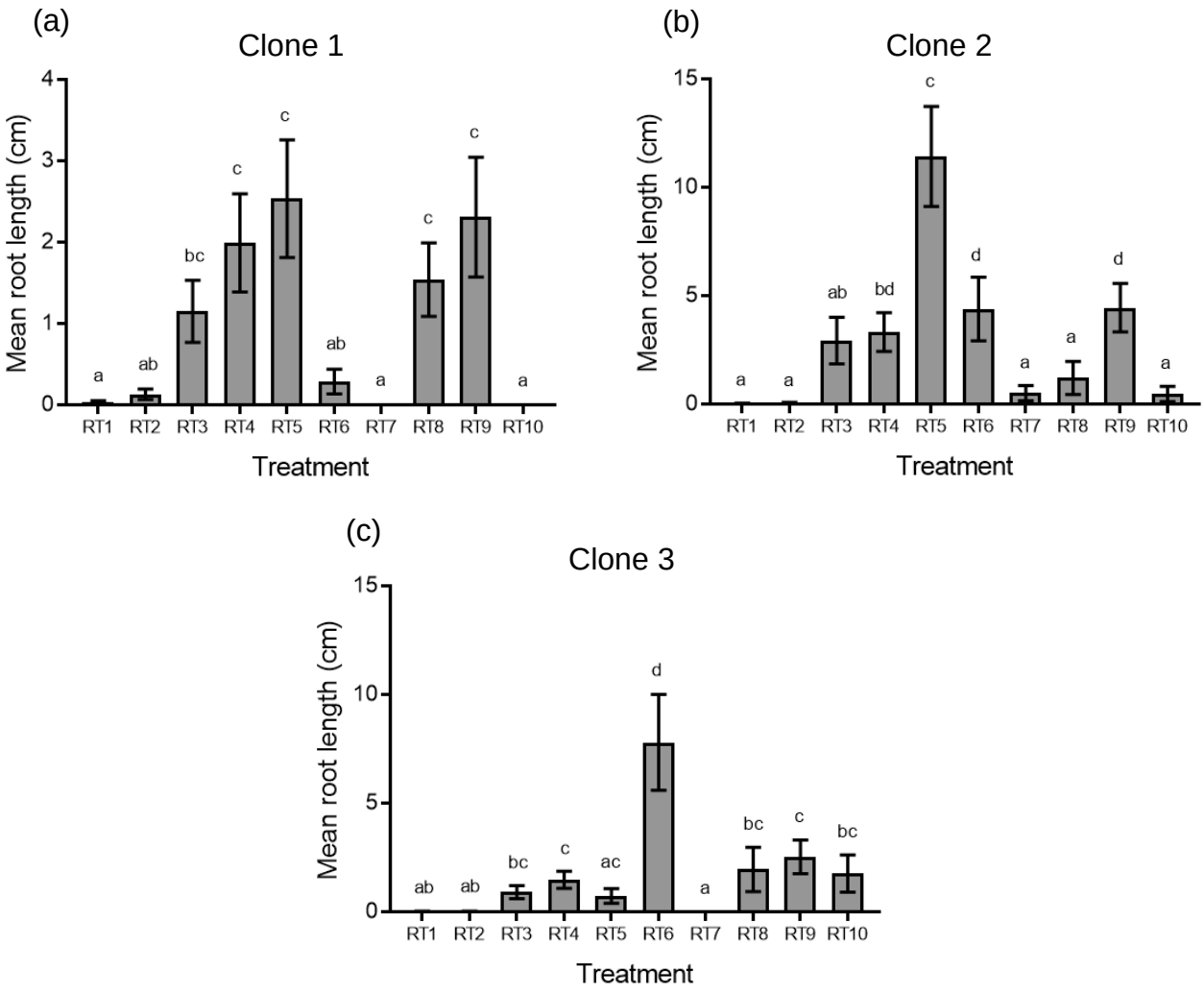

Figure 3.5 Comparison of the mean total length of the roots formed by **(a)** *Eucalyptus* clone 1; **(b)** *Eucalyptus* clone 2 and; **(c)** *Eucalyptus* clone 3, following a 28-day growth period, in ten different rooting media. The error bars represent the standard error of the mean and the letters denote whether significant differences exist between the root lengths in response to the rooting treatments tested, analyzed via two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

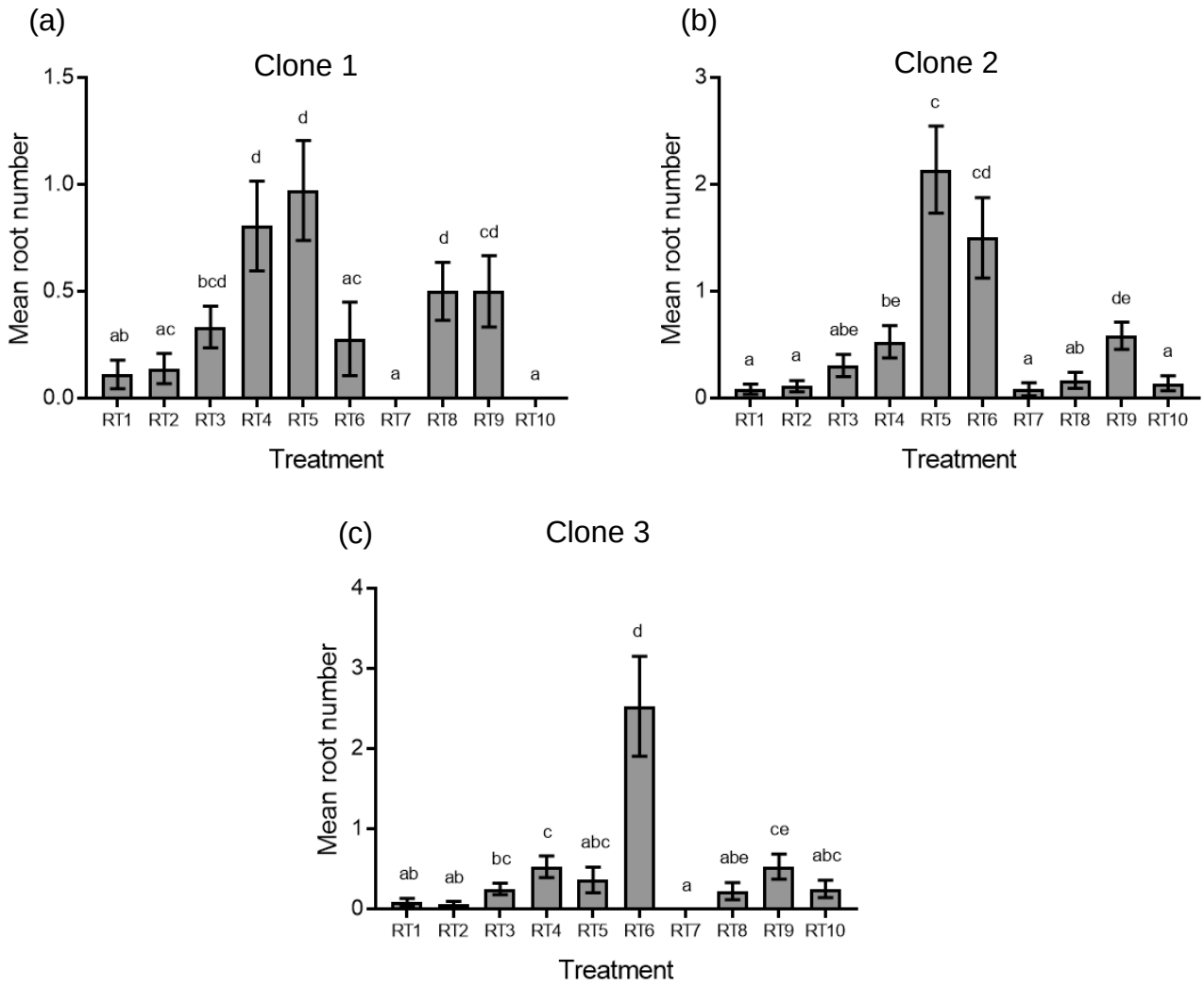

Figure 3.6 Comparison of the mean number of roots formed by **(a)** *Eucalyptus* clone 1; **(b)** *Eucalyptus* clone 2 and; **(c)** *Eucalyptus* clone 3, following a 28-day growth period, in ten different rooting media. The error bars represent the standard error of the mean and the letters denote whether significant differences exist between the root numbers in response to the rooting treatments tested, analyzed via two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

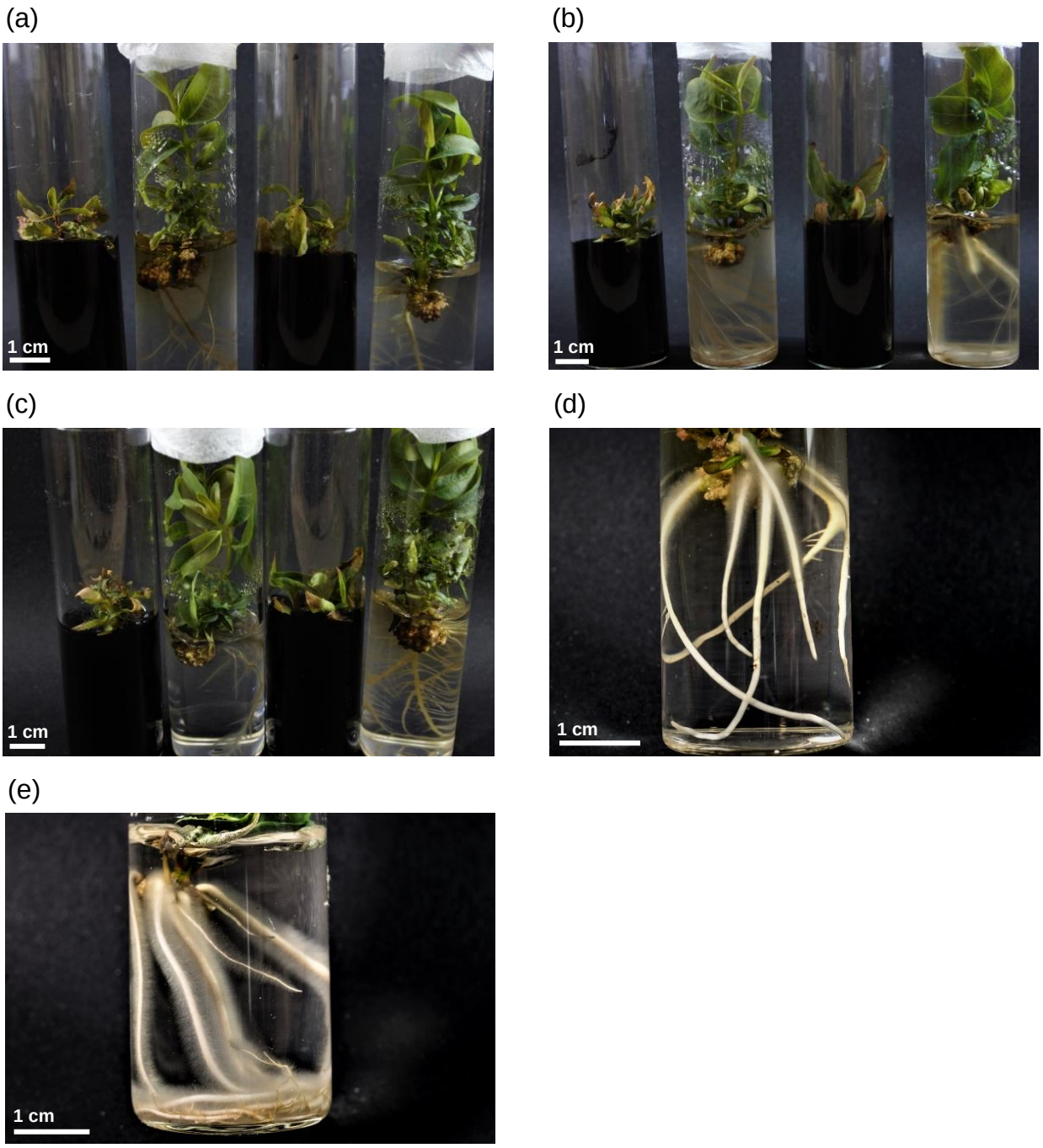

Figure 3.7 Depiction of *Eucalyptus* **(a)** clone 1; **(b)** clone 2 and; **(c)** clone 3, following a 28 d growth period, in medium containing either activated charcoal (Rt10, dark medium) or IAA with strigolactone (Rt9, transparent medium). These images illustrate the delayed senescence phenotype and shoot elongation induced by the synthetic strigolactone, GR24, in the medium. **(d)** Illustration of the phenotype lacking root hairs obtained with the 0.1 mg ℓ^{-1} IBA treatment, versus; **(e)** an enhancement in root hair production, induced by 0.5 mg ℓ^{-1} IBA treatment. The size bars represent 1 cm.

Selection of the optimal rooting treatments

The selection of the top three rooting treatments was primarily determined based upon consideration of the root number, length and percentage (Figure 3.5; 3.6; Table 3.1). However, in cases where similar rhizogenic responses were recorded, the overall explant health and phenotypic responses was considered to be an influential factor as the health of the explants may increase the survivability when acclimatizing the explants to *ex vitro* conditions. The chi squared test of independence revealed that the rooting percentage for *Eucalyptus* clone 1 was significantly influenced by the optimal treatment type (p = 0.041), this was reflected by Chi-squared result, $X^2(2, N = 108) = 6.41$. On the contrary, the rooting percentages obtained for clones 2 and 3 were not significantly influenced based on the optimal treatment type (p = 0.172 and p = 0.05), yielding Chi-squared values of $X^2(2, N = 108) = 3.52$ for clone 2 and $X^2(2, N = 108) = 5.98$ for clone 3.

The optimal rooting response induced by the natural auxin, IAA, was at a concentration of 0.5 mg ℓ^{-1} in conjunction with reduced sucrose and MS content, for each of the *Eucalyptus* clones presently tested (Figure 3.9 a; d; g). Likewise, 0.5 mg ℓ^{-1} of IBA supplemented into the growth medium, enhanced root length, number and percentage for clones 2 and 3, although this was not the case with clone 1, with the exception of root number. Despite this, the roots produced in the presence of 0.5 mg ℓ^{-1} of IBA were substantially thicker (Figure 3.9 b; e; h), with an abundance of root hairs (Figure 3.7 e), in comparison to those induced with 0.1 mg ℓ^{-1} IBA (Figure 3.7 d), for all of the clones tested. The incorporation of GR24 into the rooting medium resulted in more uniform rooting percentages, lengths and numbers across clones, when compared with IBA (Figure 3.8 a; b; Table 3.2). Therefore, the optimal treatments that were repeated in triplicate and compared between the *Eucalyptus* clones were with 0.5 mg ℓ^{-1} IAA (Rt4), 0.5 mg ℓ^{-1} BAP (Rt6) and with GR24 (Rt9).

Statistically, there were differences between the parameters assessed for the three treatments and across all three tested genotypes (Figure 3.8 a; b). Roots of the relatively recalcitrant genotype, clone 1, were significantly longer when induced in the presence of GR24 (Rt9) (3.24 cm ± 1.01) compared to those induced in the presence of IAA (Rt4) (1.66 cm ± 0.63) and additionally the highest rooting percentage of 33.4 % was obtained with GR24 for clone 1 (Table 3.2). Clone 3 displayed a significant enhancement in root length and number when treated with IBA (5.02 cm ± 1.49; 1.44 ± 0.37) compared to IAA (1.25 cm ± 0.37; 0.48 ± 0.13) (Figure 3.8 a; b). Similarly, the root number was significantly enhanced

in clone 2 in response to IBA treatment (1.29 ± 0.35) compared to IAA (0.5 ± 0.14). Furthermore, the rooting percentage for clone 2 was higher in response to IBA (44.6 %) than with IAA (30.6 %) treatment. Despite the fact that treatment with GR24 seldomly induced a significantly enhanced rooting response, this treatment was never significantly outperformed by any of the top performing treatments, for root stimulation in any of the clones tested (Figure 3.8 a; b).

In order to obtain the optimal clonal response to *in vitro* adventitious rooting, it was apparent that the different clones required different PGR types as well as concentrations. Considering this, clone 1 displayed an optimal overall rooting response in the presence of GR24 (Rt9), whereas the other clones responded optimally to the IBA (Rt6) treatment. However, when developing a protocol to fit all three clones, the optimal treatment that generated the most consistent results for inducing roots was with GR24 (Rt9), which also produced considerably healthier shoots than those cultured on the other media types without strigolactones, as these shoots displayed fewer signs of necrosis and chlorosis (Figure 3.9 a-i).

Table 3.2 Mean (x̄) rooting percentage of the top performing rooting treatments following a 4-week growth period for clones 1, 2 and 3 grown on Rt4, Rt6 and Rt9. All treatments were replicated in triplicate (n=108). The emergence of adventitious roots was determined to be dependent on the treatment type, for *Eucalyptus* clone 1 and not for clones 2 and 3, determined by the Chi-squared test of independence.

	% Rooting per treatment		
Clone	Rt4	Rt6	Rt9
1	32.5	19.6	33.4
2	30.6	44.6	43.5
3	32.5	47.2	34.3

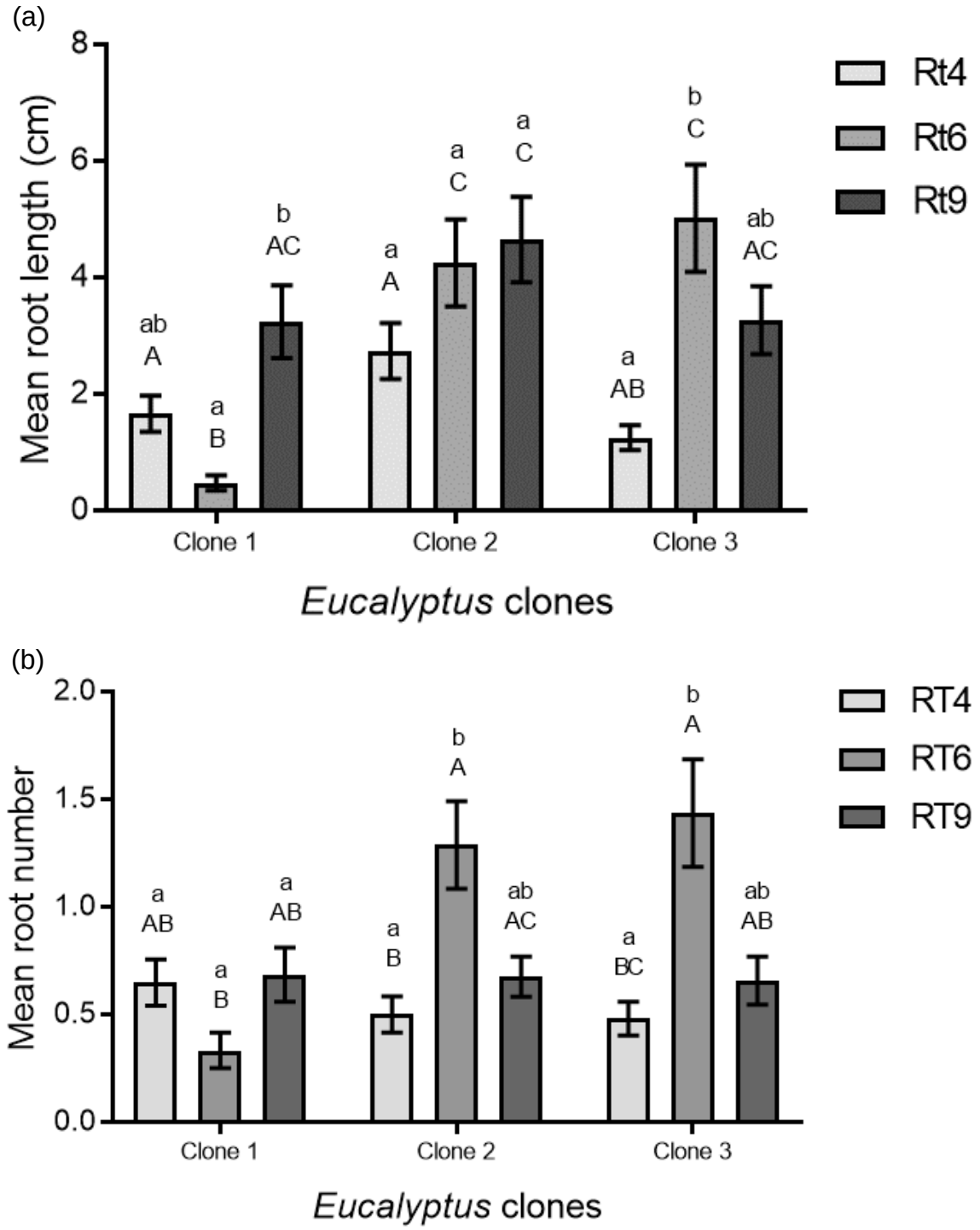

Figure 3.8 Comparison of the mean **(a)** Root length and; **(b)** Root number of three *Eucalyptus* clones, subject to the top three rooting treatments (Rt4, Rt6, Rt9), following a 28 d growth period and repeated in triplicate. The error bars represent the standard error of the mean, the lower-case letters denote whether significant differences existed between the treatments for a given clone and the upper-case letters indicate a significant difference amongst the clones, analysed via two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

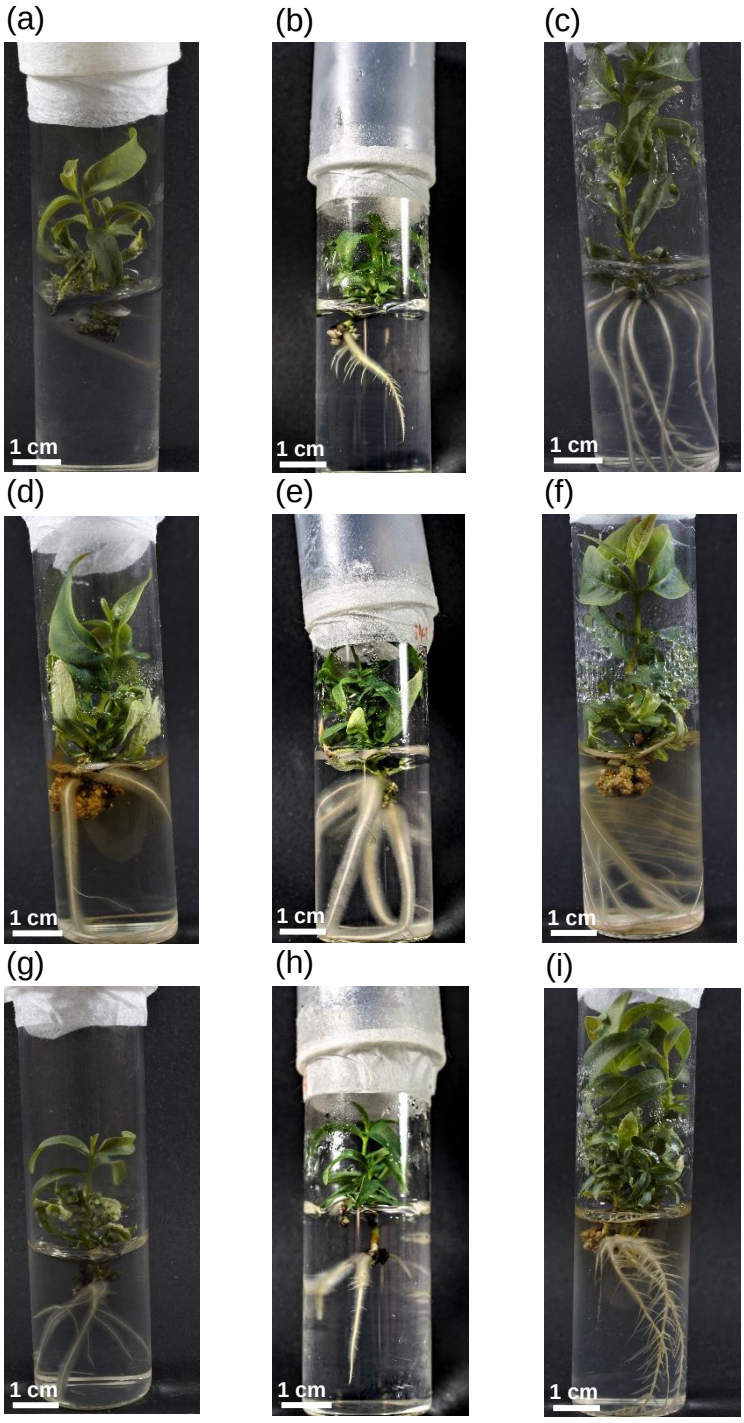

Figure 3.9 Depiction of the rooting responses induced by the top three rooting treatments on each of the *Eucalyptus* clones tested. **(a)** Rhizogenic responses elicited on *Eucalyptus* clone 1 by IAA (Rt4); **(b)** IBA (Rt6) and; **(c)** GR24 (Rt9) treatment. **(d)** The rhizogenic responses elicited on *Eucalyptus* clone 2 by IAA (Rt4); **(e)** IBA (Rt6) and; **(f)** GR24 (Rt9) treatment. **(g)** The rooting responses observed for *Eucalyptus* clone 3 upon exogenous supply of IAA (Rt4); **(h)** IBA (Rt6) and; **(i)** GR24 (Rt9).

Acclimatization of the Eucalyptus *clones*

The final stage of the micropropagation process is to acclimatize the *Eucalyptus* clones to *ex vitro* growth conditions such as soil, sun, reduced humidity and nutrient content to ultimately quantify the survivability of each clone towards growth in a soil medium. The present acclimatization procedure was successful at gradually acclimating each of the *Eucalyptus* clones. The initial response observed in certain *Eucalyptus* clones was programmed senescence of the leaves at the base of the stem, possibly in response to the water loss associated with reduced humidity in comparison to *in vitro* growth conditions (Figure 3.10 a). Near the end of acclimatisation, the eucalypts appeared to have developed trichomes on the leaf and stem surfaces, to further aid with the prevention of water loss. Minimal losses were obtained for the protocol presently tested, with a loss of only a single plant for each of the *Eucalyptus* clones, after having set eight plants per clone for acclimatization. Thus, contrasting to the other experiments tested in this investigation, there was a uniform response across all three clones for acclimatization, with a survival rate of 87.5% being observed for each clone. All plantlets that survived past day 3 continued to survive past 30 d (Figure 3.10 b; c; d).

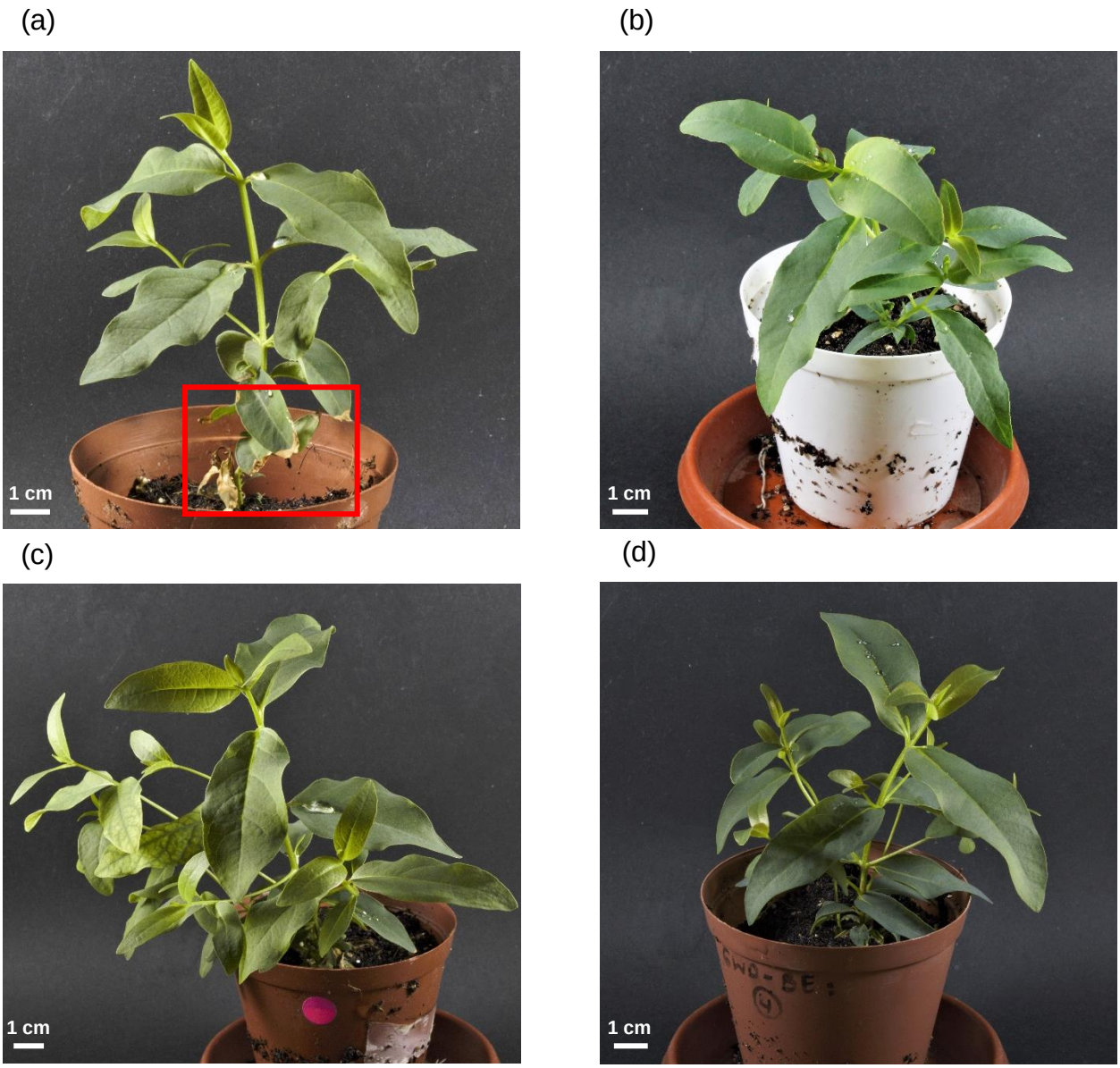

Figure 3.10 (a) Figure depicting programmed senescence of the leaves, within the red rectangle, near the base of the *Eucalyptus* plant stem to counteract the effects of desiccation; **(b)** Typical depiction of an acclimatized *Eucalyptus* plantlet for clone 1; **(c)** clone 2 and; **(d)** clone 3, following a 30 d growth period in soil.

Clonal gene expression profiles, in response to IAA

Total RNA concentration, purity and integrity

High quality RNA, with A260/A280 values ranging between 2.10 - 2.16 (da Luz et al. 2016), was obtained for all clones (Figure 3.11 a). Although the DNase treatment conducted with the Maxwell® 16 Cell LEV Total RNA Purification Kit did not completely remove all gDNA

(Figure 3.11 a), subsequent DNase treatment was able to completely remove the residual gDNA from all samples (Figure 3.11 b).

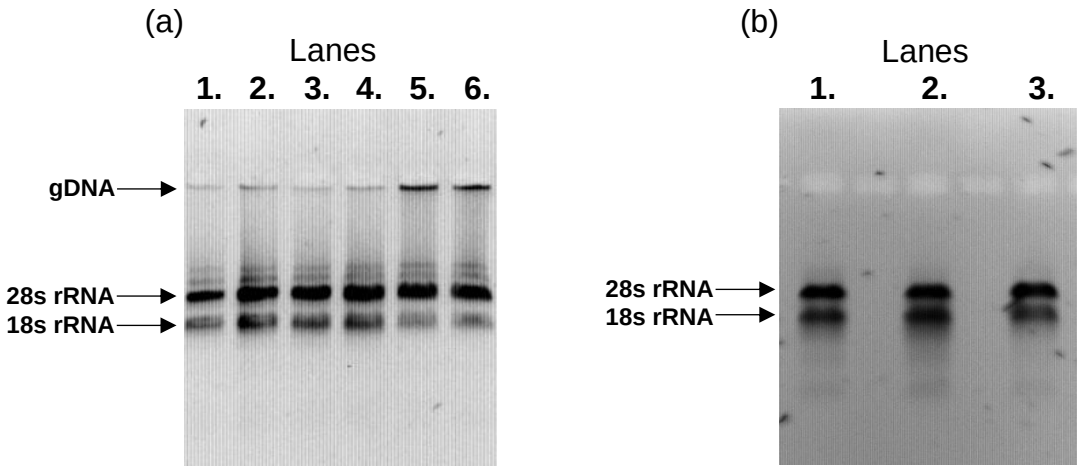

Figure 3.11 (a) Gel electrophoresis of RNA extracts from three *E. grandis* × *nitens* hybrids, each lane represents separate extractions for clone 1 (Lane: 1 & 2), clone 2 (Lane: 3 & 4) and clone 3 (Lane: 5 & 6) respectively. RNA separation was conducted on a 1% (w/v) TBE agarose gel, run at 100V. Genomic DNA (gDNA) contamination is present at the top of each well; **(b)** Gel electrophoresis of DNase treated RNA extracts from *Eucalyptus*, each lane represents separate extractions for clone 1 (Lane: 1), clone 2 (Lane: 2) and clone 3 (Lane: 3), note the complete removal of the gDNA bands from all lanes. RNA separation was conducted on a 1% (w/v) TBE agarose gel, run at 100V.

Specificity testing/validation of primers via GoTaq® PCR and melt curve

The ability of the primers to produce single amplicons for all the genes were tested, for each cDNA sample, for all of the tested *Eucalyptus* clones, via the use of GoTaq® PCR. The PCR confirmed the integrity of the cDNA, as well as the specificity of each primer set, by producing a single amplicon for the genes *PIN1*, *AUX1*, *YUC3*, *ABP1*, *H2B* and *TUA*, for all three *Eucalyptus* clones (Figure 3.12 a). Furthermore, a melt curve was generated for each of these genes, to determine the specificity of the primers during the RT-qPCR reactions. The curve revealed a single distinct peak for each of the PCR reactions, which further verified the specificity of the primers to produce a single amplicon (Figure 3.12 b).

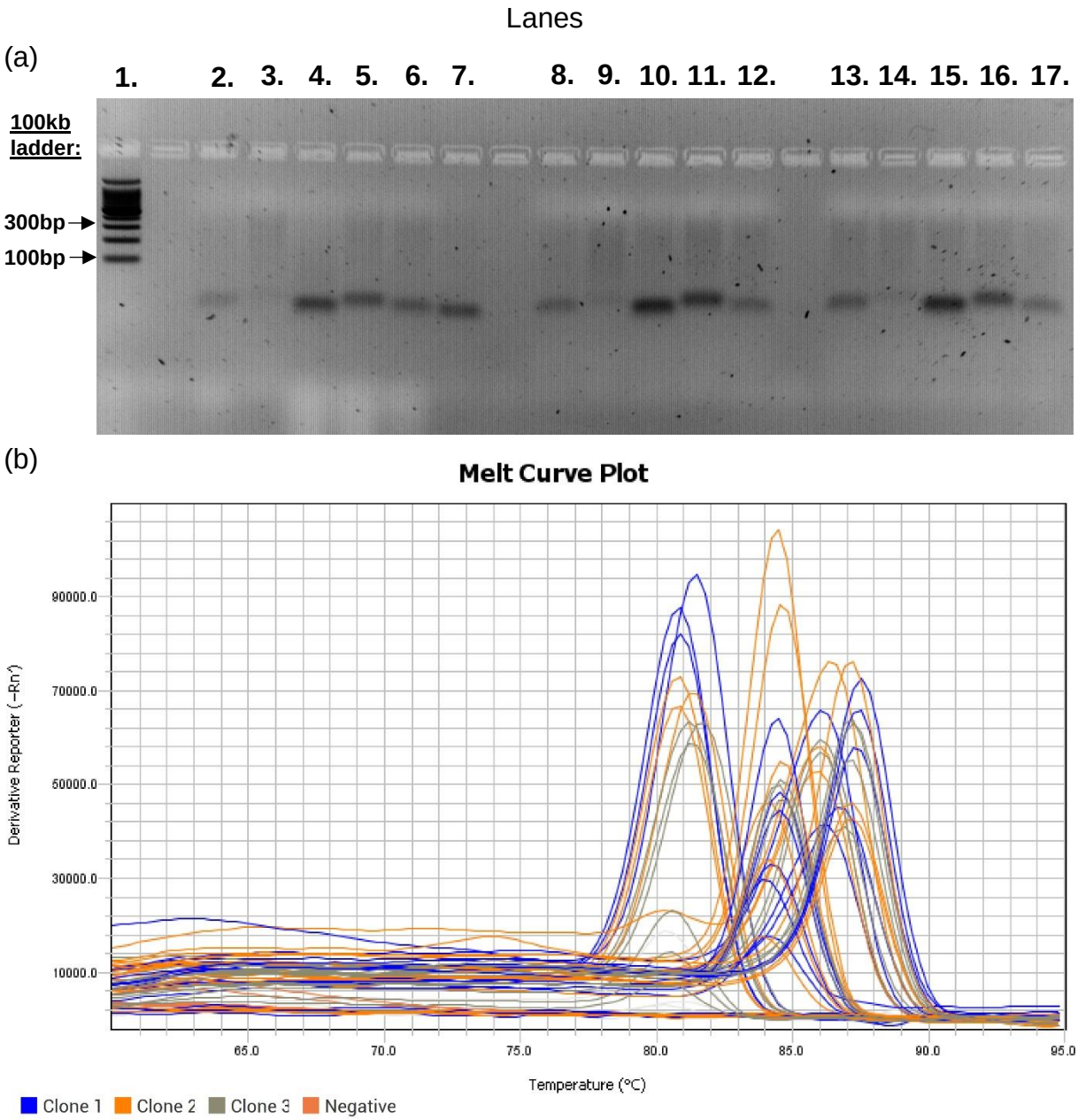

Figure 3.12 (a) GoTaq® PCR of auxin related genes to determine integrity of cDNA and to verify the presence of single amplicons, gel lanes denote amplification for; clone 1 [1. 100kb ladder, 2. *PIN1*, 3. *AUX1*, 4. *YUC3*, 5. *ABP1*, 6. *H2B*, 7. *TUA*], clone 2 [8. *PIN1*, 9. *AUX1*, 10. *YUC3*, 11. *ABP1*, 12. *H2B*], and clone 3 [13. *PIN1*, 14. *AUX1*, 15. *YUC3*, 16. *ABP*, 17. *H2B*]; **(b)** Melt curve plot representing the melting curves for *PIN1*, *AUX1*, *YUC3*, *ABP1*, *H2B*, *TUA* and the negative control, in each of the *Eucalyptus* clones. The presence of a single peak for each gene illustrates the presence of a single pure amplicon.

Gene expression observed during RT-qPCR

The Ct values generated from the RT-qPCR experiments were utilised to determine the relative gene expression of those presently under investigation, where a lower Ct value correlates to an increased level of gene expression or starting number of transcripts, as fluorescence was detected above the background noise earlier in the RT-qPCR reaction.

The RT-qPCR amplification for clone 1 revealed usable Ct values for the genes *PIN1*, *AUX1*, *H2B* and *TUA*, as the technical repeats for these genes displayed a standard deviation of 0.15, 0.44, 0.22 and 0.36 respectively. Clone 2 and clone 3 displayed a similar trend, although the technical repeats for these genes displayed a standard deviation of 0.16, 0.16, 0.17 and 0.22, for clone 1, and 0.12, 0.09, 0.3 and 0.16 for clone 3, respectively. The standard deviation between the Ct value repeats obtained for *YUC3* was 1.21 for clone 1 and 1.5 for clone 2, suggesting considerable variation between the technical repeats, rendering these Ct values unreliable. RT-qPCR was unable to detect the expression of *YUC3* for clone 3. Consequently, *YUC3* was excluded from further analysis. The RT-qPCR analysis was also unable to detect expression of *ABP1* for clone 1 or clone 2. *ABP1* expression was detected for clone 3, however, the standard deviation between the Ct values obtained revealed a standard deviation of 1.38. Consequently, *ABP1* was excluded from further analysis.

The Ct values obtained from *PIN1*, *AUX1*, *H2B* and *TUA* were highly reproducible in all three of the *Eucalyptus* clones and thus the Ct values for these genes were retained for further analysis.

Differential gene expression patterns of in vitro Eucalyptus *clones*

To further elucidate the differential phenotypic growth responses observed between the *Eucalyptus* clones during *in vitro* multiplication, elongation and rooting stages, gene expression studies were conducted to determine the influence of exogenously supplied IAA on the levels of gene expression between the clones.

The relative overall expression of *PIN1* and *AUX1* was compared among the tested *Eucalyptus* clones, to determine the levels of gene expression upon exogenous IAA stimulation. The RT-qPCR results found that clone 3 displayed the highest relative gene

expression for *PIN1*, compared to both clones 1 and 2, whereas no significant differences were observed between clones 1 and 2 for *PIN1* expression (Figure 3.13 a). In response to exogenous auxin supplementation, clone 1 revealed the lowest relative gene expression for *AUX1*, in comparison to clones 2 and 3. No significant difference was found between clone 2 and clone 3 for the relative expression of *AUX1* (Figure 3.13 a).

The relative expressions of *PIN1* to *AUX1* were then determined for each of the *Eucalyptus* clones, to identify whether an endogenous bias of *PIN1:AUX1* (efflux:influx) may exist within each clone, which may provide clues as to why the clones respond differently to various *in vitro* conditions. Clones 1 and 3 showed a *PIN1* bias over *AUX1*(Figure 3.13 b). In contrast, the relative expressions of PIN1 and AUX1 were statistically equivalent in clone 2 (Figure 3.13 b).

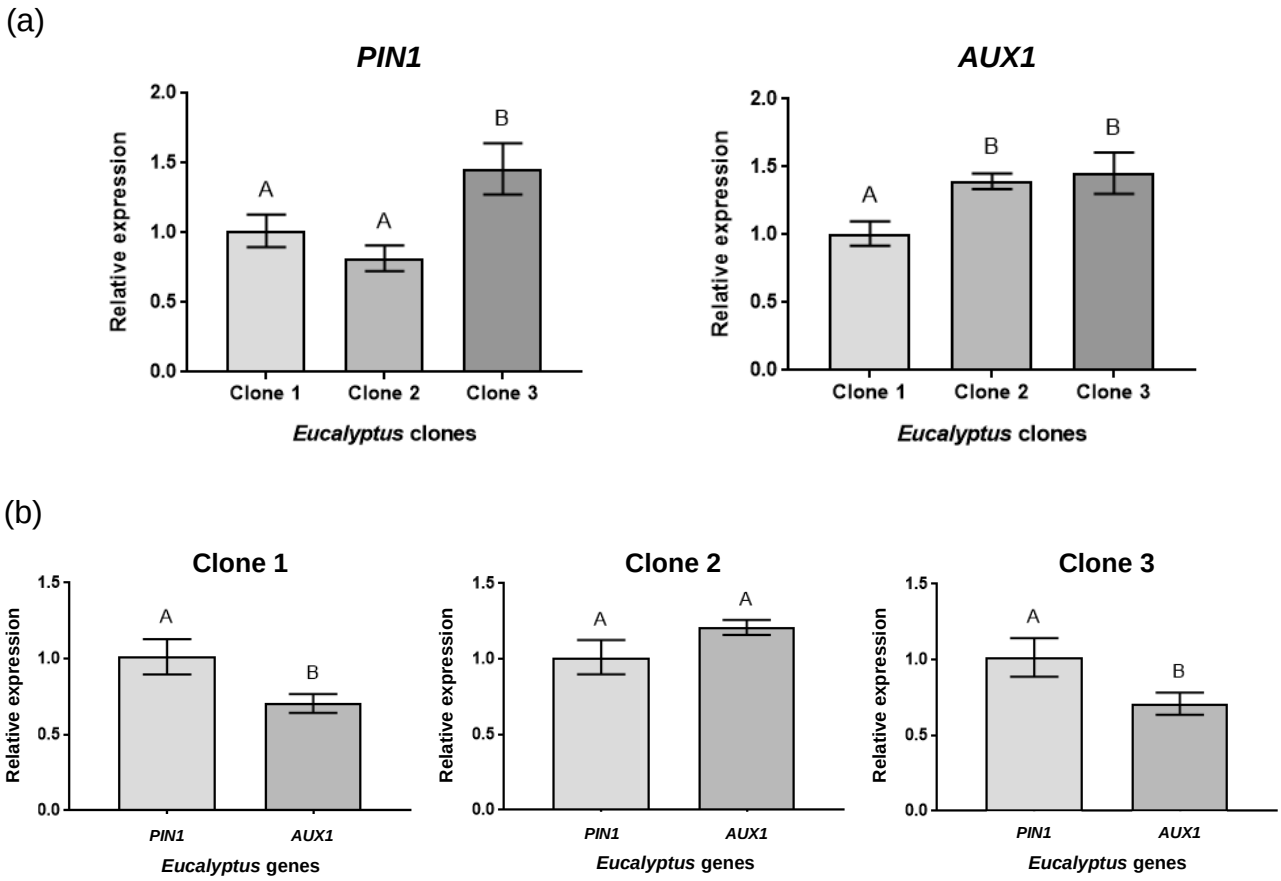

Figure 3.13 (a) Comparisons of the mean relative expression levels of *PIN1* and *AUX1*, in each *Eucalyptus* clone. Error bars represent the standard error of the mean and letters denote whether significant differences in gene expression exist between the *Eucalyptus* clones (Tukey's HSD with a 95% confidence interval [$p \leq 0.05$]). The gene expression of *PIN1* and *AUX1*, for clone 1, were used as the calibrators and are thus set at one. Therefore, the gene expressions of clones 2 and 3 are determined in relation to clone 1. **(b)** Comparisons of the mean relative expression of *PIN1* versus *AUX1*, for clone 1, clone 2 and clone 3. Error bars represent the standard error of the mean and the letters denote whether significant differences in gene expression exist between the *PIN1* and *AUX1* (Tukey's HSD with a 95% confidence interval [$p \leq 0.05$]). The *PIN1* gene expression for each of the clones tested were used as the calibrators and are thus set at one. Therefore, the expression of *AUX1* is determined in relation to *PIN1*, for each of the clones.

CHAPTER IV

Discussion

Micropropagation has proven to be an indispensable technique for the effective propagation of numerous *Eucalyptus* spp*.,* especially in large scale propagation programs that require conservation of the germplasm. Numerous factors may influence *in vitro* shoot survival and development. However, plant growth, morphogenesis and rooting are most notably impacted via the action of PGRs (George et al. 2008 a). Different *Eucalyptus* clones have been shown to respond to PGRs in a clone-specific manner, thus necessitating the development of novel protocols for each clone. The present study aimed to develop a technique to generate effective protocols for the *in vitro* multiplication, elongation and rooting of the three *E. grandis* × *nitens* clones under investigation. The results from each respective growth stage have provided insight as to the extent of the variability amongst the three *E. grandis* × *nitens* clones in response to various PGRs tested. In addition, this section elaborates on the technique employed to acclimatize the *Eucalyptus* clones, and the potential factors that may have led to the success of this technique. Lastly, the variable patterns of gene expression that were observed amongst the clones are discussed and potential parallels are drawn with the phenotypic growth responses that were presently observed in response to *in vitro* IAA supplementation.

In vitro *multiplication of* Eucalyptus grandis x nitens *explants*

Multiplication serves as a means of rapidly producing large number of viable shoot propagules, for various purposes. This shoot proliferation response is primarily under the influence of cytokinins, which reduce apical bud dominance leading to an increase in axillary bud proliferation (George et al. 2008 a). Cytokinin responses are often carried out in conjunction with auxins, each of which have an influence on different aspects of the cell growth cycle, hence the inclusion of auxins in the multiplication stage to induce a desired physiological response (Gaspar et al. 1996). Elevated cytokinin:auxin concentrations favour bud and shoot formation, and hence in the present study, multiplication was induced on media containing 0.1 mg ℓ^{-1} IAA in combination with 0.1-0.5 mg ℓ^{-1} of various cytokinins such

as BAP, kinetin and *meta*-topolin (Caraballo et al. 2010). The responses to the cytokinins yielded bud proliferation for all three tested *Eucalyptus* clones, albeit with variations among them. This observation has often been reported in the literature, which is why *in vitro* propagation protocols tend to be clone-specific (Brondani et al. 2012; Nakhooda et al. 2012; 2013; Nakooda and Jain 2016).

All clones displayed an increase in bud proliferation in response to increased BAP concentrations in the medium, until the greatest proliferation was achieved at a concentration of 0.5 mg ℓ^{-1}. Conversely, a reduction in kinetin concentration in the multiplication medium correlated with improved bud proliferation (Figure 3.2 a). This outcome has drawn parallels with various studies that report enhanced multiplication performance with the use of BAP compared to kinetin in the growth medium (Trindade et al. 1990; Muhammad et al. 2007). The observed clonal differences in response to the various cytokinin types presently tested may be explained with reference to studies that associated these variations with the rate of uptake observed in different genotypes, the rate of translocation of the cytokinin to the plant meristematic tissues, and based upon metabolic processes by which cytokinins are degraded, via cytokinin oxidase, or conjugation with amino acids or sugars to form biologically inactive compounds (Blakesley 1991; Kaminek 1992; Mok and Mok 2001).

PGR metabolism and conjugation has a significant influence on the availability of their physiologically active forms, and consequently has a major impact on cell dedifferentiation and differentiation (Klems et al. 2000). Conjugation represents a means to inactivate physiologically-active compounds such as PGRs (Kaminek 1992). The superior shoot proliferation presently observed with BAP may be attributed to a reduced tendency of BAP to form conjugates (Buah et al. 2010). Consequently, this translates into a greater presence of BAP in the free or ionized form, which will then be readily available to the *Eucalyptus* shoots, resulting in enhanced shoot proliferation *in vitro*. Similarly, observations by Klems et al. (2000) report an enhanced stability of BAP compared to other purine-type cytokinins, which are considered to be relatively chemically unstable.

In addition, the clones presently investigated displayed optimal shoot proliferation in the presence of the aromatic cytokinin *meta*-topolin. The positive effects of *meta*-topolin may be due to the hydroxyl group present in the side chains of topolins that allows for the formation of *O*-glucoside conjugates that are easily translocated and converted into free active forms, when required by the shoots (Amoo et al. 2011; Amoo and van Staden 2013). The bud

proliferation observed in response to the cytokinins BAP and *meta*-topolin were statistically similar between the clones.

Furthermore, it was found that in most cases *meta*-topolin resulted in significantly taller shoots in comparison to BAP and kinetin. Additionally, *meta*-topolin treatment resulted in shoots with larger leaves compared to BAP and kinetin, for all the clones tested, although this was not quantified. This response is supported by the results of Gentile et al. (2014), who reported that *meta*-topolin positively influences shoot growth and quality, whilst simultaneously reducing the occurrence of hyperhydric explants, compared to BAP. Although certain multiplication treatments induced a degree of shoot elongation, the incorporation of an elongation stage may still be necessary to flush out persistent PGRs, especially cytokinins, that may antagonise the rhizogenic effects of auxins utilised during rooting (Nakhooda et al. 2013). Therefore, in most cases, shoots from the multiplication stage should not be transferred directly into the rooting stage.

The present study has illustrated the extent of clonal variation that exists amongst the tested *E. grandis* × *nitens* hybrids in response to various multiplication treatments, which may be attributed to the underlying genetic composition of each respective clone. The overall bud proliferation results on all media tested indicated that clone 2 was the most responsive to the PGRs supplied *in vitro*, followed by clone 3, and lastly clone1. This finding has alluded to differences in each clone's ability to transport, perceive and metabolise exogenous PGRs.

In vitro *elongation of* Eucalyptus grandis x nitens *explants*

Many *Eucalyptus* species require the inclusion of an elongation stage to prepare shoots for rooting. This stage typically contains reduced concentrations of cytokinins compared with the preceding shoot proliferation medium (Ying-Hua Su et al. 2011). This reduction in exogenous cytokinins, coupled with an increase in auxin (compared to shoot proliferation medium) also serves to 'prime' shoots for the rooting stage that follows, since elevated cytokinins levels in shoots may preclude adventitious root formation through auxin antagonism (Gupta et al. 1983; Le Roux and van Staden 1991; Nakhooda and Jain 2016). Often, a reduction in rooting performance may be a consequence of carry-over of cytokinins (Nakhooda et al. 2012).

The auxin IAA was incorporated into the elongation medium to inhibit axillary bud formation, consequently driving apical dominance (Müller and Leyser 2011). Furthermore, substantially

lower concentrations of kinetin, BAP and *meta*-topolins, compared to those that were used for multiplication, were incorporated into the elongation medium to inhibit adventitious root formation (Durbak et al. 2012). Et1 consisted of auxin with the omission of cytokinin from the growth medium, so that the effects each cytokinin on shoot elongation could be determined. The auxin:cytokinin ratio was maintained at 10:1 respectively (Table 2.1), in all the elongation media tested, the variable responses towards these treatments was therefore attributed to the particular clone and cytokinin analogue.

As illustrated during multiplication, clones 1 and 3 responded equally poorly to exogenously supplied kinetin (Et2), resulting in a significantly reduced level of elongation compared to the other elongation treatments. On the other hand, the cytokinins BAP and *meta*-topolin were consistently amongst the top two treatments in terms of elongation, for all three clones (Figure 3.4). These findings were consistent with the multiplication experiment, where enhanced bud proliferation was induced via the use of BAP and *meta*-topolin rather than kinetin, possibly due to the greater abundance of these cytokinins in the free or ionized form (Buah et al. 2010; Amoo et al. 2011; Amoo and van Staden 2013). In combination with IAA, the presence of BAP and *meta*-topolin enhanced shoot elongation in all cases when compared to IAA in isolation, indicating a complementary relationship between these cytokinins and the auxin.

The shoot elongation observed in the *Eucalyptus* clones in the present study required both auxins and cytokinins, reflecting the complex interaction between these PGRs in regulating growth and development. IAA appeared to be the primary driver of apical dominance through an effect commonly referred to as the canalisation hypothesis, whereby sustained polar auxin export is necessary for consistent bud activity and subsequent growth (Balla et al. 2011). The consistent polar export of auxin (positive canalisation) from the apical bud results in a concentration gradient from the apical bud down the stem region, often referred to as the auxin sink; once the stem becomes saturated with auxin it loses its sink ability, thus preventing the flow of auxin from axillary buds into the stem and ultimately inhibiting axillar growth (Sachs 1969; Müller and Leyser 2011). Although auxins are required to drive apical dominance, shoot elongation is enhanced via the presence of cytokinins, which are critical to promote the activation of the cell cycle for cell division and have been shown to upregulate auxin biosynthesis locally in the bud (Werner et al. 2001; Jones et al. 2010). Thus, enhanced acropetal growth observed in the *Eucalyptus* clones may have potentially been promoted via the combined effect of cytokinins with auxins supplemented into the medium, firstly to

control cellular division (cytokinin) and secondly to target this growth to the apical bud (auxin).

Reduced shoot elongation associated with Et1, consisting solely of auxin, compared to those treatments with cytokinins is possibly in agreement with the canalisation hypothesis, such that the exogenous auxin supplied to the *in vitro* clones may have, in the absence of cytokinin, resulted in an auxin excess in the shoots, thus potentially reducing positive canalisation from the apical as well as axillar buds, ultimately reducing shoot elongation and cellular division. This observed stunted growth during cytokinin deprivation is supported by experiments by Werner et al. (2010) who observed a similar phenotypic response in transgenic tobacco plants, engineered for cytokinin oxidase expression, to reduce the endogenous cytokinin content.

Optimal elongation for all tested clones was obtained on Et4, containing the cytokinin *meta*-topolin (Figure 3.4). This may be explained by the ability of topolins to form *O*-glucoside conjugates that allow for the seamless translocation of *meta*-topolin to the apical buds of the *Eucalyptus* clones, where the activation of the cell cycle and upregulation of auxin biosynthesis can take place (Amoo et al. 2011; Amoo and van Staden 2013).

In vitro *rooting of* Eucalyptus grandis x nitens *explants*

The final critical stage of the micropropagation strategy involves the stimulation of *Eucalyptus* shoots to form adventitious roots. The rooting stage often incorporates the use of exogenously-supplied auxin as the sole PGR in the growth medium, to stimulate adventitious roots, with the omission of exogenous cytokinins as these have been shown to inhibit adventitious root formation (George et al. 2008 a; Durbak et al. 2012). Typically, the auxins IBA, IAA and NAA have been incorporated into growth medium to induce adventitious root formation in various *Eucalyptus* spp. (Nakhooda et al. 2011; Brondani et al. 2012; Girijashankar 2012). In the present investigation, various concentrations of these auxin analogues were tested in the growth medium to determine the rooting response of the *Eucalyptus* clones. Furthermore, two additional treatments using a biostimulant, BC204, and the synthetic strigolactone, GR24, incorporated into the growth medium together with IAA, were included, to determine their effects on adventitious rooting. Lastly, one rooting treatment included the addition of activated charcoal without any PGRs in the medium.

A reduction in MS salts from full strength to quarter strength, and reduction in sucrose from 20 g ℓ^{-1} to 15 g ℓ^{-1} significantly enhanced the rhizogenic response in all three tested clones. Comparable observations have been previously documented in literature, where a reduction in MS salt concentration to quarter strength has been shown to significantly enhance the induction of adventitious roots in multiple plant species *in vitro* (Dalal and Rai 2004; Pijut et al. 2012). This observation may be attributed to the reduced salinity levels in the medium resulting from the use of quarter strength MS, which has been shown to be beneficial to adventitious rooting in *E. tereticornis* (Das and Mitra 1990) and *E. grandis* hybrids (Warrag et al. 1990). The gel strength is also known to be influenced in response to medium salinity, which in turn affects the accessibility of each medium component to the explants i.e. increased gel strength in higher salinity media reduces the availability of the exogenously-supplied auxins and vitamins (George 1993). This differential gelling effect was noted during the initiation of rooting experiments, where the medium containing full strength MS had a firmer consistency than media made with quarter strength MS.

In addition to satisfying the energy demands of the *in vitro* explants, sucrose influences the osmotic potential of the medium, which also affects the uptake of components from the medium (Nourissier and Monteuuis 2008). The changes in osmotic potential, in relation to sucrose concentration, may account for the variability in rooting responses observed in different *Eucalyptus* spp., as not all genotypes will respond equally to a given osmotic potential (Cheng et al. 1992; Correa et al. 2005). Differences in clonal responses to sucrose have been reported, 10 g ℓ^{-1} sucrose resulted in successful *in vitro* rooting of *E. grandis* × *E. urophylla* (Yang et al.1995), although this same concentration resulted in an inhibitory effect on rooting for *E. urophylla* × *E. grandis* hybrids (Nourissier and Monteuuis 2008). Therefore, in the present investigation, the gel strength as well as the osmotic potential was decreased by reducing the MS and sucrose content, which has had a positive impact on adventitious rooting for the *E. grandis* × *nitens* clones.

IAA is a naturally-occurring auxin that is initially synthesised in the apical region of plants, after which it is transported basipetally via polar auxin transport to the rooting zone, where root differentiation, growth and development is stimulated (Aloni et al. 2003; Booker et al. 2003; Terasaka et al. 2005). Exogenous IAA treatment resulted in similar root length and number in all three of the *Eucalyptus* clones and increasing the concentration of this auxin from 0.1 mg ℓ^{-1} to 0.5 mg ℓ^{-1} enhanced the overall rooting percentage in all the clones. The reduced rooting ability observed with the lower IAA concentration may have arisen as a

result of an inadequate supply of IAA to the *Eucalyptus* shoots, as a substantial concentration of IAA is generally required to induce cell division and root initiation (Aloni 1980). Similar results were reported by Visser et al. (1996) and Ling et al. (2009), where a decrease in IAA concentration caused a reduction in the formation of adventitious roots. Furthermore, IAA is relatively unstable and breaks down rapidly in media, meaning that higher concentrations of this hormone may be required than for other, more stable auxins. Studies by Nissen and Sutter (1990) showed that up to 97 % of the exogenously-supplied IAA may be subject to non-biological degradation in the growth medium after a period of 20 d. In addition, IAA has a greater rate of absorption and is more easily photo-oxidised or conjugated into inactive forms than other auxins, such as IBA, that possess a superior stability and consequently have an extended persistence in plant tissues (Blakesley 1994; de Klerk et al. 1999). Therefore, the elevated IAA concentration supplemented into the growth medium may have presented more auxin in the free form, thus compensating for biological and non-biological inactivation of IAA, and elicited a prolonged effect on adventitious rooting in the *Eucalyptus* clones.

Treatment with IBA resulted in an improved rooting response in the *Eucalyptus* clones tested in comparison to IAA. Treatment with 0.1 mg ℓ^{-1} of IBA was required to yield an optimal rooting response for clone 1, while clone 3 responded optimally to 0.5 mg ℓ^{-1} of IBA. The concentration of IBA did not seem to have a significant effect on clone 2, which responded marginally better to 0.1 mg ℓ^{-1} IBA. These observations are in accordance with the notion that the *in vitro* growth responses of given *Eucalyptus* clones to a given treatment are not identical (Mankessi et al. 2009; Brondani et al. 2012). Rather, optimisation is often required per *Eucalyptus* clone. Nevertheless, the enhanced growth response of the *Eucalyptus* clones in relation to IBA may be attributed to its higher relative stability in comparison to IAA, which is more susceptible to photo-oxidation and enzymatic degradation (Ludwig-Muller 2000). Furthermore, IBA is considered to be an auxin precursor or storage form, which may be converted via fatty acid β-oxidation into free active auxin (i.e. IAA) to elicit its activity (Strader and Bartel 2011; Frick and Strader 2018). Therefore, as IBA is less susceptible to degradation, this auxin may have remained in the growth medium for an extended period of time, and via unilateral conversion to IAA, served as a steady source of an easily metabolised auxin for the *Eucalyptus* clones *in vitro* (Nissen and Sutter 1990; Forgaca and Fett-Neto 2005). This may have had a prolonged stimulatory effect on inducing adventitious roots, and thus elicited an enhanced rooting response. Similar findings have been observed

with *E. urophylla* × *grandis* hybrids, where an enhanced rooting response was obtained with IBA compared with IAA (Nourissier and Monteuuis 2008). Although two of the three *Eucalyptus* clones displayed an enhanced rooting response to a lower concentration of IBA, a drastic increase in root hairs was observed at higher concentrations of IBA, for all of the clones tested. The lack of root hairs from treatment with 0.1 mg ℓ^{-1} IBA, can lead to a drastic reduction in water and mineral nutrient uptake, which may be detrimental to survival during acclimatization (Ubalua and Nsofor 2017), especially considering that a poor survival rate during acclimatization has been previously linked to a lack of functionality of the *in vitro* derived roots (Gonclaves et al. 1998). Consequently, despite the fact that a higher IBA concentration did not elicit an improved rooting response in clones 1 and 2, the lack of root hairs may also be detrimental to acclimatisation and survival of the plants (Ubalua and Nsofor 2017).

The synthetic auxin NAA reduced the rooting performance in the *Eucalyptus* clones when compared to treatments with the natural auxins IBA and IAA. This has also been previously observed in *E. saligna* and *E. globulus* (Forgaca and Fett-Neto 2005). This response is counter-intuitive because NAA, similarly to IBA, is a very stable auxin and is thus expected to have an enhanced effect on adventitious rooting (Nissen and Sutter 1990). In certain plants, such as sugarcane, NAA is considered to be the strongest auxin in comparison to IBA and IAA (Tolera 2015). Although enhanced stability, as seen with IBA, has shown to improve rooting performance, NAA lacks the ability to be converted into easily metabolised IAA and may persist within the explants in the free form, consequently generating a super-optimal auxin content, thereby inhibiting root formation (Forgaca and Fett-Neto 2005).

To determine whether the rooting response with IAA can be enhanced, this auxin was exogenously supplemented into the growth medium at 0.5 mg ℓ^{-1} in combination with BC204 (Rt8) or GR24 (Rt9). The biostimulant BC204 has previously been demonstrated to improve plant growth, rooting of cuttings and abiotic and biotic stress responses (J Loubser pers. Comm., Stellenbosch University, 2019). BC204 did not improve the rooting response in the *Eucalyptus* clones tested, and in certain instances significantly reduced mean root length, for clone 2, and root number, for clone 3, in comparison to media containing only 0.5 mg ℓ^{-1} IAA. Furthermore, the percentage of shoots that formed adventitious roots was significantly reduced for clones 2 and 3. This indicates an inhibitory effect of this biostimulant on root formation in the presently tested *Eucalyptus* clones.

Given that strigolactones are a class of PGR (Al-Babili and Bouwmeester 2015), these play a crucial role in the regulation of cell division and elongation via hormone signalling (Hoffmann et al. 2014). In the present investigation, the strigolactone GR24 was supplied to the *Eucalyptus* shoots to determine the effect on adventitious rooting. Although no significant enhancement in rooting response was observed compared with IAA alone, the shoots of the explants treated with GR24 appeared to be taller and healthier than those of other treatments, with fewer visible signs of necrosis and chlorosis. Strigolactones, in association with auxins, have shown to positively regulate cambial activity and thus increase stem thickness by stimulation of secondary growth in *A. thaliana* and *E. globulus* (Agusti et al. 2011). Furthermore, an increase in internode length, accompanied by an inhibition of shoot branching, has been observed with the use of GR24 (Gomez-Roldan et al. 2008; de Saint Gaermain et al. 2013), which is similar to the observations of the present investigation. Strigolactones have commonly been associated with the acceleration of leaf senescence (Snowden et al. 2005) by enhancing the action of ethylene (Ueda and Kusaba 2015). Contrary to such reports, the supplementation of GR24 into the growth medium in this study resulted in a reduced prevalence of necrosis and chlorosis in the *Eucalyptus* clones when compared to treatments without strigolactone, suggesting a delay in senescence. Most studies that have demonstrated the effects of strigolactones on senescence have been reported for model plant species, such as *Arabidopsis thaliana* (Somerville and Koornneef 2002). As eucalypts are vastly different to many non-woody model plant species, it is possible that a different response in terms of senescence may occur in response to GR24 in *Eucalyptus*. This phenomenon warrants further investigation, as strigolactone treatment appears to be a promising prospect for the enhancement of *in vitro* growth for *Eucalyptus* spp.

Enhanced adventitious rooting has been reported with an auxin-free rooting medium in combination with activated charcoal (Jones and van Staden 1993). This enhancement has been related to reports that activated charcoal serves to reduce phenolic oxidation, absorb inhibitory compounds and reduces irradiance (Trueman et al. 2018). The results of the present investigation indicate that the *Eucalyptus* clones require exogenous hormone stimulation to effectively form adventitious roots, since the hormone-free rooting medium with activated charcoal drastically reduced the rooting performance in three clones.

Depending on the clone, the optimal rooting treatments in this study resulted from the incorporation of IBA at either 0.1 mg ℓ^{-1} or 0.5 mg ℓ^{-1} into the medium. However, a more

consistent rooting response was elicited amongst the clones following IAA treatment at 0.5 mg ℓ^{-1}. The incorporation of GR24, in combination with IAA, did not seem to enhance the rooting response, although the explants were visibly healthier and elongated, with reduced branching and fewer signs of necrosis. The use of IAA in combination with GR24 may therefore be a valuable generic treatment for a variety of different clones, whilst having further beneficial effects in terms of enhancing explant health.

Acclimatization of Eucalyptus grandis x nitens *plantlets*

Effective micropropagation strategies requires acclimatization to prepare plantlets grown *in vitro* for *ex vitro* establishment, to form rejuvenated clonal hedges that are useful to the forestry industry for establishing uniform forestry stands (Nakhooda and Jain 2016). Following *in vitro* proliferation, the plantlets will have an underdeveloped cuticle, thus lacking the ability to effectively regulate water loss (Beckett et al. 2013). Furthermore, during *in vitro* maintenance plants are often reared in a high nutrient environment and transfer into soil greatly reduces this nutrient availability (Padayachee et al. 2008). Consequently, acclimatization requires the gradual reduction in humidity and supplied nutrients to gradually facilitate the transition of the plantlets to grow in *ex vitro* conditions. In the present investigation, the aforementioned conditions were generated via the use of a growth chamber (Figure 2.1 f) and via the addition of ½ strength MS, which was effective at preventing the onset of desiccation and nutrient deprivation in most of the plantlets, yielding 87.5% survival (Figure 3.10 b; c; d). However, an individual plant, out of eight that were set for acclimatisation, from each of the *Eucalyptus* clones succumbed to desiccation and did not survive past day 3. Plants possess the ability to initiate induced senescence in response to biotic or abiotic stressors, which is initiated to maintain homeostasis to benefit the survival of the organism (Munné-Bosch and Alegre 2004). Plants that are vulnerable to desiccation, such as the *Eucalyptus* explants grown *in vitro*, generally initiate programmed senescence to limit the loss of water via transpiration. In addition, the nutrients from these leaves are remobilized to sustain the younger tissues as well as reproductive organs, ultimately resulting in the death of the leaf and survival of the remainder of the plant (Woo et al. 2019). Interestingly, some of the *Eucalyptus* plantlets displayed programmed senescence of the leaves near the base of the stem, possibly in an effort to preserve the apical bud and leaves from desiccation due to excessive water loss i.e. abiotic stress (Figure 3.10 a). The *Eucalyptus* plantlets developed tiny hair-like trichomes on all the stem and leaf surfaces,

which function to facilitate water retention to prevent the onset of desiccation until the plant develops additional protective mechanisms (Lusa et al. 2014). Plants grown under conditions of abundant moisture i.e. *in vitro*, often display a discontinuous cuticle layer which can be detrimental to *ex vitro* plantlet survival (Hazarika et al. 2006). Numerous studies have attempted to prevent this type of *in vitro* induced anatomy via the incorporation of a desiccant, such as media coating with oily substances (Ziv et al. 1983; Short et al. 1987), opening the growth vessel and exposing the cultures to conditions of low humidity (Brainerd and Fuchigami 1981), and by cooling the bottom of the culture vessel (Vanderschaeghe and Debergh 1988). In the present investigation, the development of the cuticle layer and trichomes were induced successfully in 87.5% of the *Eucalyptus* plantlets by a gradual reduction in humidity through the use of the acclimatization chamber designed during the study.

Gene expression studies offer clues to in vitro *performance*

In order to remain commercially competitive, the forestry industry has been in pursuit of new and innovative techniques of vegetative propagation, with micropropagation displaying the most promising approach to achieve the objective. However, each micropropagation protocol has shown disparate growth responses based on clonal response (Nakhooda and Jain 2016). These differing responses may be linked to the genetic underpinnings of the particular *Eucalyptus* clone, and as such, the study of gene expression may provide clues to the clonal differences that determine phenotypic responses to *in vitro* PGR supply.

RT-qPCR is a method which enables quantification of subtle dynamic changes in gene expression between two samples, with a high degree of sensitivity, via quantification of the mRNA transcripts present within a cell or tissue type (Chapman and Waldenström 2010). Therefore, in the present study, RT-qPCR has been employed in order to identify differences in gene expression between three *Eucalyptus* clones, to potentially uncover how these differences may correlate to the observed *in vitro* responses, potentially leading to the identification of the gene expression required for improved phenotypic responses to *in vitro* growth.

During RT-qPCR an increase in fluorescence by intercalating dye is used to determine the presence or absence of a target sequence (Rebrikov and Trofimov 2005). The cycle at which PCR amplification, and thus fluorescence, is discernible from the background

interference/noise referred to as the Ct value (Goni et al. 2009). In the present study, the *Eucalyptus* clones were exposed to IAA in all the *in vitro* growth stages, though most predominantly during elongation and rooting stages. Therefore, genes involved in polar auxin transport (*PIN1*/*AUX1*), biosynthesis (*YUC3*) and perception (*ABP1*) were analysed, to determine the cause of the variable growth responses observed in the preceding growth stages (de Almeida et al. 2015). In the present investigation, the RT-qPCR experiment was unable to accurately detect expression for *YUC3* as well as *ABP1* and as such the clonal responses towards the exogenously supplied IAA have been elucidated based upon the ability of each clone to transport this auxin throughout the plant.

Synthesis of auxins, including IAA, occurs in the shoot apex and thus efficient polar transport of these auxins is essential for growth responses in plants as this transport induces an accumulation of auxin at the regions of primordia induction that is often dependent on a local maximum for growth (Blakeslee et al. 2005). The influx of auxin into cells is modulated by members of the AUX family of transport membrane proteins, while the efflux of auxin from cells is controlled by the PIN-formed family of membrane proteins (Vanneste and Frimi 2009; Ahkami et al. 2013). During RT-qPCR, *PIN1* and *AUX1* expression was detected in all of the *Eucalyptus* clones tested, these genes were variably expressed based upon the clonal identity. Therefore, the findings of the present study propose a specific pattern of gene expression that correlates with the clonal responses observed *in vitro*, such that clone 2 was the most responsive to auxin supply *in vitro*, followed by clone 3 and lastly clone 1.

Unlike auxins such as 2,4-D and 1-NAA, IAA is transported and accumulated within plant cells via the joint action of auxin influx and efflux carriers, as seen in tobacco suspension cultures (Delbarre et al. 1995; Swarup and Péret 2012). The report by Delbarre et al. (1995) suggests that auxin accumulation is thus defined as the ratio of auxin influx to auxin efflux (A_i / A_e), which, for the present study, applies to the ratio of *AUX1* to *PIN1* transporters (*AUX1* / *PIN1*). Clone 2 displayed an equal relative gene expression for both of the auxin transporters *PIN1* and *AUX1*, which suggests that these transporters may be equally abundant within the cells of clone 2 (Figure 3.13 b). With a relatively comparable ability to facilitate auxin influx and efflux, it is suggested that auxin transport in clone 2 may be more capable of maintaining the optimum required auxin gradient, in comparison to clones 1 and 3. As a result, clone 2 responded best to the *in vitro* protocol presently tested. Thus, for clone 2, auxin influx may be presumably equivalent of auxin efflux. Conversely, clone 1 and clone 3 collectively displayed an increased relative gene expression that favours *PIN1*

(auxin efflux) over *AUX1* (auxin influx). As a result, auxin influx may not match efflux, creating an imbalance in the auxin transport requirement for growth and physiology, i.e. auxin efflux (*PIN1*) is significantly greater than influx (*AUX1*) and thus accumulation of auxin is greatly reduced (Delbarre et al. 1995). Auxin accumulation has been shown to stimulate the elongation of hypocotyls in *Brassica oleracea* (Esmon et al. 2006) as well as lateral root formation in *Arabidopsis thaliana* (Laskowski et al. 2008). Furthermore, due to a *PIN1* bias, the cells of clone 1 and clone 3 may display a rapid acropetal efflux of auxin from the medium into the plant stem, when compared to clone 2. Studies have reported a reversal of tissue polarity and subsequent repolarisation of *PIN1* proteins in tissues upon exogenous auxin supply, which has been evidenced to redirect auxin flow through plant tissues away from the site of auxin application, via the action of *PIN1* proteins (Sauer et al. 2006). This response may reduce positive canalisation via the acropetal accumulation of auxin into the stem of clone 1 and clone 3 plants, ultimately diminishing the sink strength of the stem as well as apical bud auxin outflow, in effect reducing plant growth (Sinohara et al. 2013). The model developed by Prusinkiewicz et al. (2009) draws parallels with the observations of the present study, where the subsequent inclusion or activation of a second auxin source (i.e. from the growth medium) will prevent the flow against a competing source and sink. In addition to having an equal relative expression of *PIN1*:*AUX1*, clone 2 displayed one of the significantly lowest levels of relative expression for *PIN1* (Figure 3.13 a), suggesting a slower negative/acropetal canalisation flow from the lower stem region in comparison to clone 1 and clone 3, thus for clone 2 basipetal auxin flow or positive canalisation may still be maintained to facilitate bud outgrowth (Balla et al. 2011). Although clones 1 and 3 shared the same patterns of gene expression with respect to *PIN1:AUX1*, the greater abundance of these auxin transporters in clone 3 as opposed to clone 1 (Figure 3.13 a) may have allowed for more rapid directional mediation of auxin fluxes to generate auxin maxima and thus a gradient of flow which influenced development (Stoma et al. 2008; Křeček et al. 2009; Berkel et al. 2013).

Therefore, the quantification of gene expression levels in the *Eucalyptus* clones may serve a powerful tool to potentially predict the clonal responses to *in vitro* growth conditions, especially considering the disparate responses of clones. Currently the detection of *PIN1* and *AUX1* was useful in determining a pattern of gene expression that correlated to an enhanced *in vitro* response in the *Eucalyptus grandis* × *nitens* clones. These have revealed that a balance of these transporters (i.e. equal influx and efflux) will potentially enhance

auxin transport, which has shown to improve physiology and development, most prevalent presently in clone 2. However, imbalances of these transporters, such as in clone 1, resulted in an imbalance in auxin transport, essentially reducing the overall response to exogenous IAA. In clone 3, this imbalance was marginally overcome by a greater abundance of these transporters.

CHAPTER V

Conclusion and future prospects

Research overview

The present study was undertaken with the intention of optimizing the micropropagation strategy for three commercially valuable *Eucalyptus grandis* × *nitens* clones. Micropropagation of eucalypts often requires multiple growth stages that incorporate various PGRs, to promote the differentiation of various plant tissues into organs, for the proliferation of the explants via the organogenesis route (Gaspar et al. 1996). However, a wide range of responses exist between *Eucalyptus* clones, with some proving to be more recalcitrant to vegetative propagation than others (Mankessi et al. 2009; Brondani et al. 2012). Hence, in an attempt to improve plantlet yield during micropropagation, there is a need to modify the various stages involved. The stages presently targeted for improvement were multiplication, elongation, rooting and acclimatization. Multiple treatments were tested for each respective growth stage, in order to identify the best treatment for the eucalypts tested. The findings also displayed significantly different growth responses for each respective stage, in a clone-dependent manner. Preliminary gene expression studies were therefore incorporated to determine the genotypic factors may have influenced the observed phenotypic response.

Research outcomes and prospects

Typically, micropropagation strategies have been empirical, requiring explants that are initially established into multiplication medium prior to transfer into the elongation and rooting growth stages (Jones and van Staden 1993; Brondani et al. 2012). This practice is suitable for *Eucalyptus* clones that are known to be somewhat amenable to *in vitro* growth conditions. However, for clones that are novel and require rigorous testing, this process can prove to be costly and time consuming. Therefore, the present investigation has proposed a novel approach whereby the plant material is cultured on a maintenance medium and from this

medium can be distributed directly into any growth stage of micropropagation for improvement.

The shoots of the *Eucalyptus* clones were proliferated on medium containing auxin and cytokinin, during the multiplication stage. The supplementation of *meta*-topolin at 0.5 mg ℓ^{-1} in combination with 0.1 mg ℓ^{-1} IAA was shown to produce the greatest bud proliferation, in a consistent manner between the *Eucalyptus* clones, in comparison to other PGR combinations tested. Furthermore, *meta*-topolin treatment produced larger shoots with healthier leaves as opposed to treatments with the other PGRs.

To prepare the *Eucalyptus* shoots for rooting, micropropagation strategies often incorporate an elongation stage to condition shoots for rooting (Nakhooda and Jain 2016). The *Eucalyptus* clones tested in this study were elongated via the combination of auxin and cytokinin in the growth medium. Treatment with the PGRs, *meta*-topolin at 0.1 mg ℓ^{-1} along with IAA at 0.5 mg ℓ^{-1}, elicited the greatest elongation of the *Eucalyptus* shoots for each clone, in comparison to the other PGR combinations. The findings for elongation mirror those of multiplication, such that the inclusion of *meta*-topolin into the growth medium produced shoots that had larger leaves and shoots and displayed a lower prevalence of necrosis.

The optimal rooting response for the *Eucalyptus* clones presently tested was determined by investigating different auxin and supplemental treatments. The rooting responses for clones 2 and 3 was most optimal when treated with IBA at 0.5 mg ℓ^{-1}, whereas clone 1 responded best to treatment with 0.029 mg ℓ^{-1} of the strigolactone GR24. Although the clonal responses were disparate, the most consistent treatment between the top three was IAA plus GR24. In addition, GR24 appeared to invigorate the explants *in vitro* in comparison to the other treatments tested. In future studies, the characteristics that are seemingly invigorating the explants can be quantified to determine the extent and manner in which strigolactones improve explant growth. Furthermore, the delay in senescence should be further explored as this response may allow the cultures to be maintained for extended periods of time (i.e. reducing the subculture frequency), and hence reduce expenditure, time and resources required to preserve valuable genotypes *in vitro*. Elongated shoots were also observed in the *Eucalyptus* cultures treated with strigolactones during rooting, and this may be a useful additive to replace the cytokinins in elongation which have been shown to hinder subsequent adventitious rooting (Nakhooda et al. 2013). Lastly, strigolactones in combination with IBA may be tested to determine whether an improved rooting response can be elicited.

The final stage of micropropagation often requires the proliferated plantlets to be acclimatized to a soil environment. Often care is required to prevent losses of plant material due to desiccation, contaminants and lack of nutrients (Beckett et al. 2013 Padayachee et al. 2008). Therefore, this study incorporated an acclimatization stage which maintained a humid environment, sterilised all pots and containers used and lastly supplemented the *Eucalyptus* plantlets with ½ strength MS as a source of nutrients. These conditions were gradually reduced until the plantlets were entirely self-sufficient. This was successful at acclimating 87.5% of the *Eucalyptus* plantlets to *ex vitro* conditions, for all of the clones tested.

Micropropagation strategies often require each respective growth stage to be conducted in series, with one stage succeeding the next (Jones and van Staden 1993; Brondani et al. 2012; Nakhooda et al. 2013). The present investigation has thus served to provide insight as to how each *Eucalyptus* clone responded to various PGR types and concentrations within the medium. However, the influences that each stage may have on one another, when conducted in succession, has not been tested. Therefore, to further the present findings, future research should consider investigating the effects of carry-over PGRs from one respective growth stage to the next. Previous research has provided evidence that stable PGRs incorporated into the growth media may persist in the cultured explants and ultimately lead to carry-over of PGRs into subsequent growth stages, thus eliciting adverse effects on growth (Gupta et al. 1983; Le roux and van Staden 1991; Nakhooda et al. 2012; 2013). Considering this, the best performing multiplication treatments from the present investigation should be repeated, with these plants being transferred directly into the best performing elongation and subsequently rooting treatments, without the inclusion of an intervening maintenance medium such as the one incorporated into the present investigation. The new results obtained from the various growth stages conducted in succession, should be contrasted to the results obtained from the present study, to determine whether the stable PGRs may display persistence and potentially influence growth either positively or negatively when used in succession.

Significant growth differences in response to the various treatments tested were present between the *Eucalyptus* clones. Consequently, RT-qPCR was conducted to potentially provide validation for this observed variation in response, based on clonal identity. The expression of the auxin transporters *PIN1* and *AUX1* was variable amongst the clones. Clone 2 had equal relative expression levels of *PIN1* to *AUX1*, while the other clones had

an increased relative *PIN1* expression (efflux) compared with *AUX1* (influx). The equal expression of these auxin transporters in clone 2 may suggest a balance in polar transport of IAA, which has evidently elicited a more efficient growth response compared to clones 1 and 3 (Blakeslee et al. 2005). These different patterns of relative gene expression provide genetic evidence that seemingly correlates with the findings that clone 2 responded best to *in vitro* conditions of the three *Eucalyptus* clones, followed by clone 3 and lastly clone 1.

Although the RT-qPCR experiment performed in the current investigation was unable to accurately detect expression for *YUC3* and *ABP1* for any of the *Eucalyptus* clones, future studies may accurately quantify the expression of these genes via the incorporation of reference/housekeeping genes that exhibit a lower relative gene expression than the reference genes used this study. This will enable the RT-qPCR experiment to be performed on a more undiluted cDNA sample and will thus allow for the quantification of *YUC3* and *ABP1* to fall within the limits of quantification, as the fluorescence will be detected at a much earlier cycle number (Forootan et al. 2017). The limits of quantification are determined by the minimum amount of cDNA of the target gene, present in the sample that can be quantitatively measured with an adequate degree of accuracy and precision according to the defined experimental conditions (Armbruster and Pry 2008). YUCCA proteins are responsible for the biosynthesis of the auxin, IAA, via participation in the TAA/YUC auxin biosynthetic pathway, which is presumably the main source of endogenous IAA in plants (Zheng et al. 2015). Furthermore, a reduction in IAA biosynthesis is commonly associated with a reduction in the formation of adventitious roots (Bellini et al. 2014). *ABP1* serves as an auxin receptor that is often associated with the initial responses of cell growth (Tromas et al. 2010). Auxin perception is achieved by the joint action of ABP1 with transmembrane kinases (TMK) on the cell membrane in an auxin-dependent manner (Xu et al. 2014). The active perception of auxins by *ABP1* promotes the transcription of genes associated with auxins, as well as the modification to the cell walls necessary for root development (de Almeida et al. 2015). Additionally, the study by de Almeida et al. (2015) proposed that an increased *ABP1* expression results in enhanced water uptake, thereby assisting with cellular expansion and facilitating the growth of plant organs. It would therefore be extremely valuable to investigate the expression patterns of these two genes during the rooting stage in the three different clones, to determine whether these would shed more light on the relative abilities of the clones to form adventitious roots.

The relative gene expression determined from the cDNA isolated from each *Eucalyptus* clone suggests the relative abundances of targeted proteins. However, many processes may occur between transcription and translation that may affect final expression of the particular gene, and consequently it would be valuable to quantify the absolute abundances of PIN1 and AUX1 transporter proteins via a proteomics-based approach (Vogel et al. 2010). This will provide further insight as to whether the relative gene expression of these genes is equivalent to protein translation within these *Eucalyptus* clones (Vogel and Marcotte 2012).

This study has broad applications that are not only restricted to fundamental research, but have potential application in the genetic improvement of genotypes, propagation of commercially-valuable cultivars and elevating the yield of commercially-valuable forestry species, not only limited to *Eucalyptus*. This research is a fundamental step in the development of silvicultural techniques to offer a high yielding, yet sustainable source for microcuttings, to meet the worldwide demands placed on the forestry industry and its products, while simultaneously alleviating the stresses placed upon natural forests and accompanying ecosystems.

Literature Cited

Aggarwal D, Kumar A, Reddy MS. 2010. Shoot organogenesis in elite clones of *Eucalyptus tereticornis*. Plant Cell Tissue and Organ Culture 201: 45-52.

Aggarwal D, Kumar A, Sharma J, Reddy MS. 2012. Factors affecting micropropagation and acclimatization of an elite clone of *Eucalyptus tereticornis* Sm. *In Vitro* Cellular and Developmental Biology-Plant 48: 521-529.

Agusti J, Herold S, Schwarz M, Sanchez P, Ljung K. 2011. Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. Proceedings of the National Academy of Sciences of the United States of America 108: 20242–20247.

Ahad A, John E, Maqbool A, Malik K. 2018. Development of efficient micropropagation system for *E. camaldulensis* with respect to age of explants. Pakistan Journal of Agricultural Sciences 55: 23-27.

Ahkami AH, Melzer M, Ghaffari MR, Pollmann S, Javid MG, Shahinnia F, Hajirezaei MR, Druege U. 2013. Distribution of indole-3-acetic acid in *Petunia hybrida* shoot tip cuttings and relationship between auxin transport, carbohydrate metabolism and adventitious root formation. Planta 238: 499-517.

Ahmad A, Anis M. 2019. Meta-topolin improves *in vitro* morphogenesis, rhizogenesis and biochemical analysis in *Pterocarpus marsupium Roxb*: a potential drug-yielding tree. Journal of Plant Growth Regulation 38: 1007-1016.

Ahuja MR. 1993. Biotechnology and clonal forestry. In: Ahuja MR, Libby WJ (ed.), *Clonal Forestry* I*: Genetics and Biotechnology*. Berlin: Springer. pp135-144.

Akiyoshi DE, Klee HJ, Amasino RM, Nester EW, Gordon MP. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Plant Pathogenic Bacteria 81: 377-387.

Al-Babili S, Bouwmeester HJ. 2015. Strigolactones, a novel carotenoid-derived plant hormone. Annual Review of Plant Biology 66: 161-186.

Aloni R, Schwalm K, Langhans M, Ullrich CI. 2003. Gradual shifts in sites of free auxin production during leaf primordium development and their role in vascular differentiation and leaf morphogenesis in *Arabidopsis*. Planta 216: 841-853.

Aloni R. 1980. Role of auxin and sucrose in the differentiation of sieve and tracheary elements in plant tissue cultures. Planta 150: 255-263.

Amoo SO, Finnie JF, van Staden J. 2011. The role of *meta*-Topolins in alleviating micropropagation problems. Plant Growth Regulation 63: 197-206.

Amoo SO, van Staden J. 2013. Influence of plant growth regulators on shoot proliferation and secondary metabolite production in micropropagated *Huernia hystrix*. Plant Cell Tissue and Organ Culture 112: 249-256.

Ana M, Vieitez M, Vieitez L. 1980. Culture of chestnut shoots from buds *in vitro*. Journal of Horticultural Science 55: 83-84.

Armbruster DA, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clinical Biochemistry Reviews 29: S49-S52.

Arruda SCC, Souza GM, Almeida M, Gonçalves AN. 2000. Anatomical and biochemical characterization of the calcium effect on *Eucalyptus urophylla* callus morphogenesis *in vitro*. Plant Cell Tissue and Organ Culture 63: 143-154.

Baccarin FJB, Brondani GE, de Almeida LV, Vieira IG, de Oliveira LS, de Almeida M. 2015. Vegetative rescue and cloning of *Eucalyptus benthamii* selected adult trees. New Forests 46*:* 465-483.

Balla J, Kalousek P, Reinohl V, Friml J, Prochazka S. 2011. Competitive canalization of PIN-dependent auxin flow from axillary buds' controls pea bud outgrowth. Plant Journal 65: 571-577.

Baltimore MD. 2000. The *Eucalyptus*: A natural and commercial history of the Gum tree. Environmental History 7: 341-342.

Bandyopadhyay S, Cane K, Rasmussen G, Hamill JD. 1999. Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species - *Eucalyptus nitens* and *E. globulus*. Plant Science 140: 189-198.

Barrueto Cid LP, Machado ACMG, Carvalheira SBRC, Brasileiro ACM. 1999. Plant regeneration from seedling explants of *Eucalyptus grandis x E. urophylla*. Plant Cell Tissue and Organ Culture 56: 17-23.

Beckett RP, Marschall M, Laufer Z. 2013. Hardening enhances photoprotection in the moss *Atrichum androgynum* during rehydration by increasing fast- rather than slow-relaxing quenching. Journal of Bryology 27: 7-12.

Bell DT, van der Moezel PG, Bennett IJ, McComb JA, Wilkins CF, Marshall SCB, Morgan AL. 1993. Comparisons of growth of *Eucalyptus camaldulensis* from seeds and tissue culture: root, shoot and leaf morphology of 9-month-old plants grown in deep sand and sand over clay. Forest Ecology and Management 57: 125-139.

Bellini C, Pacurar DI, Perrone I. 2014. Adventitious roots and lateral roots: similarities and differences. Annual Review of Plant Biology 65: 639-666.

Benett BM. 2010. The El Dorado of Forestry: The *Eucalyptus* in India, South Africa, and Thailand, 1850-2000. International Review of Social History 55: 27-50.

Bhatia S. 2015. Tissue culture. In: Bhatia S, Sharma K, Dahiya R, Bera T (ed.), *Modern applications of plant biotechnology in pharmaceutical sciences*. London: Academic press. pp 31-107.

Blakeslee JJ, Peer WA, Murphy AS. 2005. Auxin transport. Current Opinion in Plant Biology 8: 494-500.

Blakesley D. 1991. Uptake and metabolism of 6-benzyladenine in shoot cultures of *Musa* and *Rhododendron*. Plant Cell Tissue and Organ Culture 25: 69-74.

Blakesley D. 1994. Auxin metabolism and adventitious root formation. In: Davis TD, Haissig BE (ed.), *The biology of adventitious root formation*. New York: Plenum Press. pp 143–153.

Blakesley D, Kiernan RJ. 2001. Cryopreservation of axillary buds of a *Eucalyptus grandis x Eucalyptus camaldulensis* hybrid. Cryo-Letters 22: 13-18.

Booker J, Chatfield S, Leyser O. 2003. Auxin acts in xylem-associated or medullary cells to mediate apical dominance. Plant Cell 15: 495-507.

Brainerd KE, Fuchigami LH. 1981. Acclimatization of aseptically cultured apple plants to low humidity. Journal of the American Society for Horticultural Science 106: 515-518.

Brondani GE, De Oliveira LS, Konzen ER, Da silva ALL, Costa JL. 2018. Mini-incubators improve the adventitious rooting performance of *Corymbia* and *Eucalyptus* microcuttings according to the environment in which they are conditioned. Anais Da Academia Brasileira De Ciencias 90: 2409-2423.

Brondani GE, de Wit Ondas HW, Baccarin FJB, Gonçalves AN, de Almeida M. 2012. Micropropagation of *Eucalyptus benthamii* to form a clonal micro-garden. *In Vitro* Cellular and Developmental Biology 48: 478-487.

Brondani GE, Dutra LF, Wendling I, Grossi F, Hansel FA, Araujo MA. 2011. Micropropagation of a *Eucalyptus* hybrid (*Eucalyptus benthamii* x *Eucalyptus dunnii*). Acta Scientiarum-Agronomy 33: 655-663.

Brown AW, Kaiser KA, Allison DB. 2018. Issues with data and analyses: Errors, underlying themes, and potential solutions. Proceedings of the National Academy of Sciences of the United States of America 115: 2563-2570.

Bryant PH, Trueman SJ. 2015. Stem anatomy and adventitious root formation in cuttings of *Angophora*, *Corymbia* and *Eucalyptus*. Forests 6: 1227-1238.

Buah JN, Danso E, Taah KJ, Abole EA, Bediako EA, Asiedu J, Baidoo R. 2010. The effects of different concentrations cytokinins on the *in vitro* multiplication of planta in (*Musa sp*.). Biotechnology 9: 343-347.

Bunn E, Senaratna T, Sivasithamparam K, Dixon KW. 2005. *In vitro* propagation of *Eucalyptus phylacis* L. Johnson and K. Hill., A critically endangered relict from Australia. *In Vitro* Cellular and Developmental Biology-Plant 41: 812-815.

Bustin SA. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29: 23-39.

Butcher PA, Skinner AK, Gardiner CA. 2005. Increased inbreeding and inter-species gene flow in remnant populations of the rare *Eucalyptus benthamii*. Conservation Genetics 6: 213-226.

Caraballo MG, Oramas GG, García2 SA, Cruz EA, Bravo KQ, Caligari PDS, García-González R. 2010. Management of auxin-cytokinin interactions to improve micropropagation protocol of henequen (*Agave fourcroydes Lem*.). Chilean Journal of Agricultural Research 70: 545-551.

Carlson MC. 1938. The formation of nodal adventitious roots in *Salix cordata.* American Journal of Botany 25: 721-725.

Chapman JR, Waldenstrom J. 2015. With reference to reference genes: A systematic review of endogenous controls in gene expression studies. Plos One 10: 1-18.

Cheng B, Peterson CM, Mitchell RJ. 1992. The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon*. Plant Science 87: 207-214.

Correia D, Gonçalves AN, do Couto HTZ, Ribeiro MC. 1995. Efeito do meio de cultura líquido e sólido no crescimento e desenvolvimento de gemas de *Eucalyptus grandis* × *Eucalyptus urophylla* na multiplicação *in vitro*. Instituto de Pesquisas e Estudos Florestais 48: 107-116.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiology 139: 5-17.

da Luz AC, Pretti IR, Batitucci MDCP. 2016. Comparison of RNA extraction methods for *Passiflora edulis* sims leaves. Revista Brasileira de Fruticultura 38: 226-232.

Dalal NV, Rai VR. 2004. *In vitro* propagation of *Oroxylum indicum* Vent. a medicinally important forest tree. Journal of Forest Research 9: 61-65.

Das T, Mitra GC. 1990. Micropropagation of *Eucalyptus tereticornis* Smith. Plant Cell Tissue and Organ Culture 22: 95–103.

de Almeida MR, de Bastiani D, Gaeta ML, de Araújo Mariath JE, de Costa F, Retallick J, Nolan L, Tai HH, Strömvik MV, Fett-Neto AG. 2015. Comparative transcriptional analysis provides new insights into the molecular basis of adventitious rooting recalcitrance in *Eucalyptus*. Plant Science 239: 155-165.

de Assis TF, Fett-Netto AG, Alfenas AC. 2004. Current techniques and prospects for the clonal propagation of hardwoods with emphasis on *Eucalyptus*. In: Walter C, Carson M (ed.), *Plantation forest biotechnology for the 21st century*. Kerala: Research signpost. pp 303-333.

de Fossard RA, Nitsch C, Cresswell RJ, Lee ECM. 1973. Tissue and organ culture of *Eucalyptus*. New Zealand Journal of Forestry Science 4: 267-278.

de Klerk G-J, van der Krieken W, de Jong JC. 1999. Review: The formation of adventitious roots: New concepts, new possibilities. *In Vitro* Cellular and Developmental Biology-Plant 35: 189-199.

de Oliveira LS, Brondani GE, Batagin-Piotto KD, Calsavara R, Gonçalves AN, de Almeida M. 2015. Micropropagation of *Eucalyptus cloeziana* mature trees. Australian Forestry 78: 219-231.

de Oliveira Y, Adamuchio LG, de Oliveira C, Degenhardt-Goldbach J, Gerhardt I, Filho JCB, Dibax R, Quoirin M. 2011. IUFRO tree biotechnology conference. In: de Oliveira Y (ed.), *Indirect organogenesis from leaf explants of Eucalyptus benthamii x Eucalyptus dunnii and shoot multiplication.* Arraial d'Ajuda: BMC Proceedings 5 P145.

de Saint Gaermain A, Ligerot Y, Dun EA, Pillot JP, Ross JJ. 2013. Strigolactones stimulate internode elongation independently of gibberellins. Plant Physiology 163: 1012–1025.

Debergh PC, Read PE. 1991. Micropropagation technology and application. In: Debergh PC, Zimmerman RH (ed.), *Micropropagation*. Dordrecht: Springer. pp1-13.

Dibax R, Deschamps C, Filho JC, Vieira LG, Molinari HB, Campos MK, Quoirin MG. 2010. Organogenesis and *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus saligna* with *P5CS* gene. Biologia Plantarum 54: 6-12.

Driver JA, Kuniyuki AH. 1984. *In vitro* propagation of Paradox Walnut rootstock. Horticultural Science 19: 507-509.

Du Toit B. 2007. Effects of site management on growth, biomass partitioning and light use efficiency in a young stand of *Eucalyptus grandis* in South Africa. Forest Ecology and Management 255: 2324-2336.

Durbak A, Yao H, Mc Steen P. 2012. Hormone signaling in plant development. Current Opinions in Plant Biology 15: 92-96.

DWAF. 2004. Department of Water Affairs and Forestry. In: Grobler DC, Ntsaba M (ed.), *Strategic framework for national water resource quality monitoring programmes.* Pretoria: DWAF. 61pp.

DWAF. 2008. Department of Water Affairs and Forestry. In: Forestry Economics Services CC (ed.), *Report on commercial timber resources and primary roundwood processing in South Africa: 2006/2007*. Pretoria: DWAF. 115pp.

Enders TA, Strader LC. 2015. Auxin activity: Past, present, and future. American Journal of Botany 102: 180-196.

Esmon CA, Tinsley AG, Ljung K, Sandberg G, Hearne LB, Liscum E. 2006. A gradient of auxin and auxin-dependent transcription precedes tropic growth responses. Proceedings of the National Academy of Sciences-USA 103: 236-241.

Faeth SH, Fagan WF. 2002. Fungal endophytes: Common host plant symbionts but uncommon mutualists. Integrative and Comparative Biology 42: 360-368.

Fantini M Jr, Garaça MEC. 1989. A micropropagation system for *Eucalyptus dunnii* × *Eucalyptus sp.* Annales Des Sciences Forestieres 46: 136-139.

FAO. 2015. United Nations, Food and Agriculture Organisation. In: Mac Dicken K, Jonsson O, Pina L, Marklund L, Maulo S, Contessa V, Adikari Y, Garzuglia M, Lindquist G, Reams G, D'Annunzio R (ed.), *Global forest resources assessment*. Rome: FAO. 44pp.

Fehér A. 2019. Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology? Frontiers in Plant Science 10: 536.

Fehér A, Pasternak TP, Dudits D. 2003. Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue and Organ Culture 74: 201-228.

Fernando SC, Goodger JQD, Gutierrez SS, Johnson AAT, Woodrow IE. 2016. Plant regeneration the indirect organogenesis and genetic transformation of *Eucalyptus polybractea* R.T. Baker. Industrial Crops and Products 86: 73-78.

Fett-Neto AG, Fett JP, Veira Goulart LW, Pasquali G, Termignoni RR, Ferreira AG. 2001. Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. Tree physiology 21: 457-464.

Fishel MF. 2006. Plant growth regulators. Pesticide information office, Florida cooperative extension service, Institute of Food and Agricultural Sciences, University of Florida. Document PI-102.

Forgaça CM, Fett-Neto AG. 2005. Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance. Plant Growth Regulation 45: 1-10.

Forootana A, Sjöbackc R, Björkmanc J, Sjögreenb B, Linzd L, Kubistac M. 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomolecular Detection and Quantification 12: 1-6.

Franclet A, Boulay M. 1982. Micropropagation of frost resistant *Eucalyptus* clones. Australian Forest Research 13: 83 – 89.

Frick EM and Strader LC. 2018. Roles for IBA-derived auxin in plant development. Journal of Experimental Botany 69: 169-177.

Froldi G, Gallo U, Ragazzi E, Caparrotta L. 1999. 6-Benzylaminopurine: a plant derived cytokinin inducing positive inotropism by P2-purinoceptors. Planta Medica 65: 245–249.

FSA. 2007. South African Forestry Facts 2005/06. In: Forestry South Africa (ed.), Pietermaritzburg. 11pp.

Fu BJ, Wang YF, Lu YH, He CS, Chen LD, Song CJ. 2009. The effects of land-use combinations on soil erosion: a case study in the Loess Plateau of China. Progress in Physical Geography 33: 793-804.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50: 151-158.

Gantait S, Kundu S, Ali N, Sahu NC. 2015. Synthetic seed production of medicinal plants: a review on influence of explants, encapsulation agent and matrix. Acta Physiologiae Plantarum 37: 1-12.

Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA. 1996. Plant hormones and plant growth regulators in plant tissue culture. *In Vitro* Cellular and Developmental Biology-Plant 32: 272-289.

Gentile A, Gutierrez MJ, Martinez J, Frattarelli A, Nota P, Caboni E. 2014. Effect of *meta*-Topolin on micropropagation and adventitious shoot regeneration in *Prunus* rootstocks. Plant Cell Tissue and Organ Culture 118: 373-381.

George EF. 1993. Plant Propagation by Tissue Culture, 2nd Edition. In: George EF (ed.), *Part 1, the Technology*. Edington: Exegetics Limited. 574pp.

George EF, Hall MA, de Klerk GJ. 2008 a. Plant propagation by tissue culture 3rd Edition. In: George EF, Hall MA, de Klerk GJ (ed.), *Plant growth regulators II: Cytokinins, their analogues and antagonists.* Dordrecht: Springer. pp 205-227.

George EF, Hall MA, de Klerk GJ. 2008 b. Plant propagation by tissue culture 3rd Edition. In: George EF, Hall MA, de Klerk GJ (ed.), *Plant tissue culture procedure-background.* Dordrecht: Springer. pp 1-28.

Girijashankar V. 2012. *In vitro* regeneration of *Eucalyptus camaldulensis*. Physiology and Molecular Biology of Plants 18: 79-87.

Godsmark R, Oberholzer F. 2017. South African forestry and forest products industry 2016. Forestry South Africa. pp 1-162.

Gomes F, Canhoto JM. 2003. Micropropagation of *Eucalyptus nitens* Maiden (Shining Gum). In Vitro Cellular and Developmental Biology-Plant 39: 316-321.

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pages V, Dun EA. 2008. Strigolactone inhibition of shoot branching. Nature 455: 189–194.

Gonclaves JC, Diogo G, Amâncio S. 1998. *In vitro* propagation of chestnut (*Castanea sativa* and *C. crenata*): Effects of rooting treatments on plant survival, peroxidase activity and anatomical changes during adventitious root formation. Scientia Horticulturae 72: 265-275.

Gorst JR, Slaytor M, De Fossard RA. 1983. The Effect of Indole-3-Butyric Acid and Riboflavin on the Morphogenesis of Adventitious Roots of *Eucalyptus ficifolia* F. Muell. Grown *In Vitro*. Journal of Experimental Botany 34: 1503-1515.

Gupta PK, Mehta UJ, Mascarenhas AF. 1983. A tissue culture method for rapid clonal propagation of mature trees of *Eucalyptus torelliana* and *Eucalyptus camaldulensis*. Plant Cell Reports 2: 296-299.

Hajari E, Watt MP, Mycock DJ, McAlister B. 2006. Plant regeneration from induced callus of improved *Eucalyptus* clones. South African Journal of Botany 72: 195-201.

Haroldsen MV, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB, Powell ALT. 2012. Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. Plant sciene 3: 1-12. Hartmann HT, Kester DE, Davies Jr. FT, Geneve RL. 2011. Hartmann and Kester's plant propagation, 8th edition.

In: Davies FT, Geneve RL, Kester DE, Hartmann HT (ed.), *Plant propagation: principles and practices*. São Paulo: Prentice-Hall. 915pp.

Hazarika BN, Teixeira da Silva J, Talukdar A. 2006. Ornamental and Plant Biotechnology Volume II. In: Teixeira da Silva J (ed.), *Effective acclimatization of in vitro cultured plants: Methods, physiology and genetics*. UK: Global Science Books. pp 427–438.

Hill RS, Truswell EM, Mcloughlin S, Dettmann ME. 1999. Evolution of the Australian flora: Fossil evidence. Australian Journal of Botany 2: 251-320.

Hinchee M, Rottmann W, Mullinax L, Zhang C, Chang S, Cunningham M, Pearson L, Nehra N. 2010. Short-Rotation Woody Crops for Bioenergy and Biofuels Applications. *In Vitro* Cellular and Developmental Biology-Plant 45: 619-629.

Ho CK, Chang SH, Tsay JY, Tsai CJ, Chiang VL, Chen ZZ. 1998. *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants. Plant Cell Reports 17: 675-680.

Hoffmann B, Proust H, Belcram K, Labrune C, Boyer FD. 2014. Strigolactones inhibit *caulonema* elongation and cell division in the moss *Physcomitrella patens*. PLOS ONE 9: e99206.

Huang ZC, Zeng FH, Lu XY. 2010. Efficient regeneration of *Eucalyptus urophylla* from seedling-derived hypocotyls. Biologia Plantarum 54(1): 131-134.

Huetteman CA, Preece JE. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue and Organ Culture 33: 105-119.

Hung CD, Dung CD. 2015. Production of chrysanthemum synthetic seeds under non-aseptic conditions for direct transfer to commercial greenhouses. Plant Cell Tissue and Organ Culture 122: 639-648.

Hung CD, Trueman SJ. 2011. Alginate encapsulation of shoot tips and nodal segments for short-term storage and distribution of the eucalypt *Corymbia torelliana* × *C. citriodora*. Acta Physiologiae Plantarum 34:117-128.

Hung CD, Trueman SJ. 2011. Encapsulation technology for short-term preservation and germplasm distribution of the African mahogany *Khaya senegalensis*. Plant Cell Tissue and Organ Culture 107: 397-405.

Hung CD, Trueman SJ. 2011. Topophysic effects differ between node and organogenic cultures of the eucalypt *Corymbia torelliana* × *C. citriodora.* Plant Cell Tissue and Organ Culture 104: 69-77.

Ikenganyia E, Anikwe MAN, Omeje E, Adinde J. 2017. Plant Tissue Culture Regeneration and Aseptic Techniques. Asian Journal of Biotechnology and Bioresource Technology 1: 1-6.

Ipekci Z, Gozukirmizi N. 2003. Direct somatic embryogenesis and synthetic seed production from *Paulownia elongate*. Plant Cell Reports 22: 16-24.

Ishii H, Ford ED. 2001. The role of epicormic shoot production in maintaining foliage in old *Pseudotsuga menziesii* (Douglas-fir) trees. Canadian Journal of Botany 79: 251 -264.

James DJ. 1983. Adventitious root formation "*in vitro*" in apple rootstocks (*Malus pumila*) II. Uptake and distribution of indole-3yl-acetic acid during the auxin sensitive phase in M.9 and M.26. Physiologia Plantarum 57: 154–158.

Jones NB, Staden JV. 1994. Micropropagation and establishment of *Eucalyptus grandis* hybrids. South African Journal of Botany 60: 122-126.

Joshi L, Bisht P, Sharma VK, Uniyal DP. 2003. *In vitro* clonal propagation of mature *Eucalyptus* F1 hybrid (*Eucalyptus tereticornis* Sm. x *E. grandis* Hill ex. Maiden). Silvae Genetica 52: 110-113.

Jovanovic T, Arnold R, Booth T. 2000. Determining the climatic suitability of *Eucalyptus dunnii* for plantations in Australia, China and Central and South America. New Forests 9: 215-226.

Junghans DT, Alfenas AC, Brommonschenkel SH, Oda S, Mello EJ, Grattapaglia D. 2003. Resistance to rust (Puccinia psidii Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. Theoretical and Applied Genetics 108: 175-180.

Klaminek M.1992. Progress in cytokinin research. Trends in Biotechnology 10: 159-164.

Klemš M, Balla J, Machackova I, Eder J, Prochazka S. 2000. The uptake and metabolism of ^{3}H-benzylaminopurine in tobacco (*Nicotiana tabacum* L.) and cucumber (*Cucumis sativus* L.) explants. Plant Growth Regulation 31: 135-142.

Křeček P, Skůpa P, Libus J, Naramoto S, Tejos R, Friml J, Zažímalová E. 2009. The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biology 10: 249.

Laskowski M, Grieneisen VA, Hofhuis H, ten Hove CA, Hogeweg P, Maree AFM, Scheres B. 2008. Root system architecture from coupling cell shape to auxin transport. Plos Biology 6: 2721-2735.

Le Roux JJ, van Staden J. 1991. Micropropagation and tissue culture of *Eucalyptus*-a review. Tree Physiology 9: 435-477.

Leslie A, Purse J. 2016. Species with forestry potential in the British Isles. Quarterly Journal of Forestry 110: 88-97.

Leva A, Petruccelli R, Rinaldi L. 2012. Somaclonal variation in tissue culture: A case study with olive. Biotechnology 6: 54.

Li J, Han X, Wang C, Qi W, Zhang W, Tang L, Zhao X. 2017. Validation of suitable reference genes for RT-qPCR data in *Achyranthes bidentata* blume under different experimental conditions. Frontiers in Plant Science 8: 1-12.

Li SW, Xue L, Xu S, Feng H, An L. 2009. Mediators, genes and signalling in adventitious rooting. Botanical Review 75: 230-247.

Ling APK, Kok KM, Hussein S, Ling S. 2009. Effects of plant growth regulators on adventitious roots induction from different explants of *Orthosiphon stamineus*. Am-Eurasian Journal of Sustainable Agriculture 3: 493–501.

Lloyd G, McCown BH. 1981. Commercially-feasible micropropagation of Mountain Laurel, Kalmia *latifolia*, by shoot tip culture. Proceedings in International Plant Propagators Society 30: 421-427.

Lovarelli D, Fusi A, Pretolani R, Bacenetti J. 2018. Delving the environmental impact of roundwood production from poplar plantations. Science of the total environment 645*:* 646-654.

Lovell PH, White J. 1986. New root formation in plants and cuttings: Developments in plant and soil sciences. In: Jackson M.B. (eds), *Anatomical changes during adventitious root formation*. Dordrecht: Springer. pp 111-140.

Ludwig-Mu¨ller JL. 2000. Indole-3-butyric acid in plant growth and development. Plant Growth Regulation 32: 219–230.

Luo J, Gould JH. 1999. *In vitro* shoot-tip grafting improves recovery of cotton plants from culture. Plant Cell Tissue and Organ Culture 57: 211-213.

Lusa MG, Cardoso EC, Machado SR, Apezzato-da-Gloria B. 2014. Trichomes related to an unusual method of water retention and protection of the stem apex in an arid zone perennial species. AoB Plants 7: plu088.

Machado SR, Marcati CR, Lange de Morretes B, Angyalossy V. 2005. Comparative Bark Anatomy of Root and Stem in *Styrax Camporum* (*Styracaceae*). IAWA Journal 26:477-487.

Makhzoum A, Yousefzadi M, Malik S, Gantet P, Tremouillaux-Guiller J. 2015. Strigolactone biology: genes, functional genomics, epigenetics and applications. Critical Reviews in Biotechnology 37: 151-162.

Makouanzi G, Bouvet JV, Denis M, Saya A, Mankessi F, Vigneron P. 2014. Assessing the additive and dominance genetic effects of vegetative propagation ability in *Eucalyptus*—influence of modeling on genetic gain. Tree Genetics and Genomes 10: 1243-1256.

Mankessi F, Saya A, Baptiste C, Nourissier S, Monteuuis O. 2009. *In vitro* rooting of genetically related *Eucalyptus urophylla* x *Eucalyptus grandis* clones in relation to the time spent in culture. Trees 23: 931-940.

Marjamaa K, Kukkola EM, Fagerstedt KV. 2007. Lignification in development. International journal of plant developmental biology 1: 160-169.

Mascarenhas AF, Hazara S, Potdar U, Kulkarni DK, Gupta PK. 1982.Proceedings of the 5th International Congress on Plant Tissue and Cell Culture. In: Fujiwara A (ed.), *Rapid clonal multiplication of mature forest trees through tissue culture.* Tokyo: Japanese association for plant tissue culture. pp 719-720.

Mc Mahon DE, Vergutz L, Valadares SV, da Silva IR, Jackson RB. 2019. Soil nutrient stocks are maintained over multiple rotations in Brazilian *Eucalyptus* plantations. Forest Ecology and Management 448: 364-375.

Meier AR, Saunders MR, Michler CH. 2012. Epicormic buds in trees: a review of bud establishment, development and dormancy release. Tree Physiol*ogy* 32: 565–584.

Merkle SA, Nairn CJ. 2005. Hardwood tree biotechnology. *In Vitro* Cellular and Developmental Biology-Plant 41: 602-619.

Mineo L. 1990. Plant tissue culture techniques, Tested studies for laboratory teaching. Association for Biology Laboratory Education 11: 151-174.

Mok DW, Mok MC. 2001. Cytokinin metabolism and action. Annual Review of Plant Physiology and Plant Molecular Biology 52: 89-118.

Mokotedi ME, Watt MP, Pammenter NW. 2009. Analysis of differences in field performance of vegetatively and seed-propagated *Eucalyptus* varieties I: survival and leaf gas exchange. Southern Forests 71: 267-271.

Mokotedi MEO, Watt MP, Pammenter NW. 2000. *In vitro* rooting and subsequent survival of two clones of a cold-tolerant *Eucalyptus grandis* x *E. nitens* hybrid. Hortscience 35: 1163-1165.

Mott B, Alford RA, Schwarzkopf L. 2010. Tropical reptiles in pine forests: assemblage responses to plantations and plantation management by burning. Forest Ecology and Management 259: 916-925.

Muday GK, de Long A. 2001. Polar auxin transport: controlling where and how much. Trends in Plant Science 6: 535-542.

Muller D, Leyser O. 2011. Auxin, cytokinin and the control of shoot branching. Annuals of Botany 107: 1203-1212.

Munné-Bosch S, Alegre L. 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. Functional Plant Biology 31: 203-216.

Muralidharan EM, Mascarenhas AF. 1987. *In vitro* plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora.* Plant Cell Reports 6: 256-259.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Myburg AA, Potts BM, Marques CM, Kirst M, Gion JM, Grattapaglia D, Grima-Pettenatti J. 2007. Genome mapping and molecular breeding in plants. In: Kole C (ed.), *Forest trees*. Berlin: Springer. pp 116-160.

Naidu D, Jones N. 2009. The effect of cutting length on the rooting and growth of subtropical *Eucalyptus* hybrid clones in South Africa. Southern Forests 71: 297-301.

Nakhooda M, Jain SM. 2016. A review of *Eucalyptus* propagation and conservation. Propagation of Ornamental Plants 16: 101-119.

Nakhooda M, Mandiri E. 2016. Using synergistic exogenous phytohormones to enhance somatic embryogenesis from leaf explants of a *Eucalyptus grandis* clone. Southern Forests 78: 73-80.

Nakhooda M, Rambaran N, Mokotedi MEO, Watt MP. 2014. Sensitivity and response of *Eucalyptus grandis* × *E. nitens* cuttings to auxin treatment for rooting is consistent with, and can be predicted by *in vitro* morphogenesis. Journal of Horticultural Science and Biotechnology 89: 215-220.

Nakhooda M, Watt MP, Mycock D. 2011. Auxin stability and accumulation during *in vitro* shoot morphogenesis influences subsequent root induction and development in *Eucalyptus grandis.* Plant Growth Regulation 65: 263-271.

Nakhooda M, Watt MP, Mycock D. 2012. The properties and interaction of auxins and cytokinins influence rooting of shoot cultures of *Eucalyptus.* African Journal of Biotechnology 100: 16568-16578.

Nakhooda M, Watt MP, Mycock D. 2013. The choice of auxin analogue for *in vitro* root induction influences post-induction root development in *Eucalyptus grandis.* Turkish Journal of Agriculture and Forestry 37: 258-266.

Nissen SJ, Sutter EG. 1990. Stability of IAA and IBA in nutrient medium to several tissue culture procedures. Horticultural Science 2: 800-802.

Nourissier S, Monteuuis O. 2008. *In vitro* rooting of two *Eucalyptus urophylla X Eucalyptus grandis* mature clones. *In Vitro* Cellular and Developmental Biology-Plant 44: 263-272.

Nugent G, Chandler SF, Whiteman PH, Stevenson TW. 2001. Somatic embryogenesis in *Eucalyptus globulus*. Plant Cell Tissue and Organ Culture 67: 85-88.

Oberschelp GP, Gonçalves AN. 2016. Assessing the effects of basal media on the *in vitro* propagation and nutritional status of *Eucalyptus dunnii* Maiden. *In Vitro* Cellular and Developmental Biology-Plant 52: 28-37.

Oberschelp GP, Gonçalves AN, Meneghetti EC, Graner ÉM, Almeida MD. 2015. *Eucalyptus dunnii* Maiden plant regeneration via shoot organogenesis on a new basal medium based on the mineral composition of young stump shoots. *In Vitro* Cellular and Developmental Biology-Plant 51: 626-636.

Owen DL, Zel DW. 2000. South African forestry handbook. In: Owen DL (ed.), *Trees, forests and plantations in Southern Africa.* Pretoria: Southern African Institute of Forestry. pp 3-8.

Pacurar DI, Perrone I, Bellini C. 2014. Auxin is a central player in the hormone cross talks that control adventitious rooting. Physiologia plantarum 151: 83-96.

Padayachee K, Watt M, Edwards NM, Mycock DJ. 2009. Cryopreservation as a tool for the conservation of *Eucalyptus* genetic variability: concepts and challenges. Southern Forests 71: 165-170.

Padayachee K, Watt MP, Edwards N, MyCock DJ. 2008. Physiological responses of *Eucalyptus in vitro* axillary buds to cryopreparative desiccation and osmotic preculture: Effects of abscisic acid. South African Journal of Botany 74: 639-646.

Paek KY, Chakrabarty D, Hahn EJ. 2005. Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tissue and Organ Culture 81: 287-300.

Pallett RN, Sale G. 2004. The relative contributions of tree improvement and cultural practice toward productivity gains in *Eucalyptus* pulpwood stands. Forest Ecology and Management 193: 33-43.

Pan MJ, van Staden J.1998. The use of charcoal in *in vitro* culture – a review. Plant growth regulation 26: 155-163.

Park JD, Zheng W. 2012. Human exposure and health effects of inorganic and elemental mercury. Journal of Preventative medicine and Public Health 45: 344-352.

Pereira MJ. 2009. Reversion to juvenility: the use of epicormics in the micropropagation of mature wild shrubs of *Vaccinium cylindraceum* Smith (Ericacaeae). Life and Marine Sciences 26: 63-68.

Phillips GC, Garda M. 2019. Plant tissue culture media and practices: an overview. *In Vitro* Cellular and Developmental Biology 55: 242-257.

Pijut PM, Beasley RR, Lawson SS, Palla KJ, Stevens ME, Wang Y. 2012. *In vitro* propagation of tropical hardwood tree species—a review (2001–2011). Propagation of Ornamental Plants 12: 25–51.

Pinto G, Araujo C, Santos C, Neves L. 2013. Plant regeneration by somatic embryogenesis in *Eucalyptus* spp.: current status and future perspectives. Southern Forests 75: 59-69.

Pinto G, Santos C, Neves L, Araújo. 2002. Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Reports 21: 208-213.

Pinto GM, Park Y, Neves LP, Araujo CA, Santos C. 2008. Genetic control of somatic embryogenesis induction in *Eucalyptus globulus* Labill. Plant Cell Reports 27: 1093-1101.

Prakash MG, Gurumurthi K. 2010. Effects of type of explant and age, plant growth regulators and medium strength on somatic embryogenesis and plant regeneration in *Eucalyptus camaldulensis.* Plant cell tissue and organ culture 100: 13-20.

Prusinkiewicz P, Crawford S, Smith RS, Ljung K, Ongaro, Leyser O. 2009. Control of bud activation by an auxin transport switch. Proceedings of the National Academy of Sciences of the United States of America 106: 17431-17436.

Rademacher W. 2015. Plant Growth Regulators: Backgrounds and Uses in Plant Production. Journal of Plant Growth Regulation 34: 845-872.

Rai MK, Asthana P, Singh SK, Jaiswal VS, Jaiswal U. 2009. The encapsulation technology in fruit plants-a review. Biotechnology Advances 27*:* 671-679.

Rawat MS, Emmanuel CJSK, Uniyal DP. 1994. Macropropagation in forestry species. Indian Forester 120: 124-137.

Rayle DL, Cleland RE. 1992. The acid growth theory of auxin-induced cell elongation is alive and well. Plant physiology 99: 1271-1274.

Rebouças EL, Costa JJ, Passos MJ, Passos JRS, van den Hurk R, Silva JRV. 2013. Real Time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. Brazilian Archives of Biology and Technology 56: 143-154.

Rebrikov DV, Trofimov DY. 2006. Real-Time PCR: A review of approaches to data analysis. Applied Biochemistry and Microbiology 42: 455-463.

Reddy MC, Sri K, Murthy RA, Pullaiah T. 2012. Synthetic seeds: A review in agriculture and forestry. African Journal of Biotechnology 11: 14254-14275.

Rihan HZ, Kareem FM, El-Mahrouk ME, Fuller MP. 2017. Artificial Seeds (Principle, Aspects and Applications). Agronomy 7: 71.

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH. 1999. Cytokinin activation of *Arabidopsis* cell devision through a D-type cyclin. Science 283: 1541-1544.

Sachs T. 1969. Polarity and the introduction of organized vascular tissues. Annuals of Botany 33: 263-275.

Samantaray S, Bishoyi AK, Maiti S. 2013. Plant regeneration from callus cultures of *Vitex trifolia* (Lamiales: Lamiaceae): a potential medicinal plant. Revista de Biologia Tropical 61: 1083-1094.

Sauer M, Balla J, Luschnig C, Wisniewska J, Reinohl V, Friml J, Benkova E. 2006. Canalization of auxin flow by *Aux*/IAA-*ARF*-dependent feedback regulation of *PIN* polarity. Genes and Development 20: 2902-2911.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3: 1101-1108.

Schulze RE. 2008. South African Atlas of Climatology and Agrohydrology. In: Schulze RE (ed.). Pretoria: Water Research Commission. Section 18.3.

Schwambach J, Fadanelli C, Fett-Neto AG. 2005. Mineral nutrition and adventitious rooting in microcuttings of *Eucalyptus globulus*. Tree physiology 25: 487-494.

Scott DF, Gush MB. 2017. Forest management and the impact on water resources: a review of 13 countries. In: Garcia-Chevesich PA, Neary DG, Scott DF, Benyon RG, Reyna T (ed.), *Forest management and water in the Republic of South Africa*. París: UNESCO. pp 159-166.

Sein CC, Mitlöhner R. 2011. *Eucalyptus urophylla* S.T. Blake: ecology and silviculture in Vietnam. In: Sein CC, Mitlöhner R (ed.). Bogor: Centre for International Forestry Research. pp 2-6.

Shanthi K, Bachpai VKW, Anisha S, Ganesan M, Anithaa RG, Subashini V, Chakravarthi M, Sivakumar V, Yasodha R. 2015. Micropropagation of *Eucalyptus camaldulensis* for the

production of rejuvenated stock plants for microcuttings propagation and genetic fidelity assessment. New Forests 46: 357-371.

Sharma SK, Ramamurthy V. 2000. Micropropagation of 4-year-old elite *Eucalyptus tereticornis* trees. Plant cell reports 19: 511-518.

Shinohara N, Taylor C, Leyser O. 2013. Strigolactone can promote or inhibit shoot branching by triggering rapid depletion of the auxin efflux protein *PIN1* from the plasma membrane. Plos Biology 11: e1001474.

Short KC, Warburton, J, Roberts AV. 1987. *In vitro* hardening of cultured cauliflower and *chrysanthemum* to humidity. Acta Horticulturae 212: 329-340.

Snowden KC, Simkin AJ, Janssen BJ, Templeton KR, Loucas HM. 2005. The Decreased apical dominance1/Petunia hybrida *CAROTENOID CLEAVAGE DIOXYGENASE8* gene affects branch production and plays a role in leaf senescence, root growth, and flower development. Plant Cell 17: 746–59.

Somerville C, Koornneef M. 2002. A fortunate choice: the history of *Arabidopsis* as a model plant. Nature Reviews Genetics 3: 883-889.

Stanturf J, Vance ED, Fox TR, Kirst M. 2013. *Eucalyptus* beyond Its Native Range: Environmental Issues in Exotic Bioenergy Plantations. Journal of Forest Research 2013: 1-5.

Steffens B, Rasmussen A. 2016. The Physiology of Adventitious Roots. Plant Physiology 170: 603-617.

Stoma S, Lucas M, Chopard J, Schaedel M, Traas J, Godin C. 2008. Flux-based transport enhancement as a plausible unifying mechanism for auxin transport in meristem development. Plos Computational Biology 4: e1000207.

Stone E, Stone M. 1943. Dormant Buds in Certain Species of *Pinus*. American Journal of Botany 30: 346-351.

Strader LC, Bartel B. 2011. Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. Molecular Plant 4: 477–486.

Su YH, Liu YB, Zhang XS. 2011. Auxin–Cytokinin interaction regulates meristem development. Molecular Plant 4: 616-625.

Subbaiah MM, Minocha SC. 1990. Shoot regeneration from stem and leaf callus of *Eucalyptus tereticornis.* Plant Cell Reports 9: 370-373.

Swarup R, Peret B. 2012. *AUX/LAX* family of auxin influx carriers- an overview. Frontiers in Plant Science 3: 225.

Tanaka H, Dhonukshe P, Brewer PB, Friml J. 2006. Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cellular and Molecular Life Sciences 63: 2738-2754.

Taylor B, Powell A. 1982. Isolation of plant DNA and RNA. Focus 4: 4–6.

Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. 2010. A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines. Methods 50: S1-S5.

Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam S. 2005. PGP4, an ATP Binding Cassette P-glycoprotein, Catalyzes Auxin Transport in *Arabidopsis thaliana* Roots. Plant Cell 17: 2922-2939.

Ting CH, Mycock DJ, Padayachee K. 2014. Cold pre-treatment amplifies the responses of *in vitro Eucalyptus grandis* shoots to crypreparative drying. Cryo-Letters 35: 54-62.

Titon M, Xavier A, Otoni W. 2006. Clonal propagation of *Eucalyptus grandis* using the mini-cutting and micro-cutting techniques. Science Forum 71: 109-117.

Tolera B. 2016. Effects of naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) on *in vitro* rooting of sugarcane (*Saccharum officinarum L*) micro shoots. Journal of Biotechnology and Biomaterials 6: 1-5.

Trindade H, Ferreira JG, Pais MS, Aloni R. 1990. The role of cytokinin and auxin in rapid multiplication of *Eucalyptus* globulus grown *in vitro*. Australian Forestry 53: 221-223.

Trindade H, Pais MS. 1997. *In vitro* studies on *Eucalyptus globulus* rooting ability. *In Vitro* Cellular and developmental Biology-Plant 33: 1-5.

Trindade H, Pais MS. 2003. Meristematic nodule culture: a new pathway for *in vitro* propagation of *Eucalyptus globulus*. Trees 17: 308-315.

Tromas A, Paponov I, Perrot-Rechenmann C. 2010. Auxin binding protein 1: functional and evolutionary aspects. Trends in Plant Science 15: 436-446.

Trueman SJ, Hung CD, Wendling I. 2018. Tissue Culture of *Corymbia* and *Eucalyptus*. Forests 9: 1-42.

Trumbore S, Brando P, Hartmann H. 2015. Forest health and global change. Science 349: 814-818.

Turnbull JW. 1999. Eucalypt plantations. New Forests 17: 37-52.

Ubalua AO, Nsofor GC. 2017. The role of supporting substrates in *ex vitro* acclimatization and growth of tissue cultured cassava plantlets. Plant Knowledge Journal 6: 1-6.

Ueda H, Kusaba M. 2015. Strigolactone regulates leaf senescence in concert with ethylene in *Arabidopsis*. Plant Physiology 169: 138-147.

van Berkel K, de Boer RJ, Scheres B, ten Tusscher K. 2013. Polar auxin transport: models and mechanisms. Development 140: 2253-2268.

Vanderschaeghe AM, Debergh PC. 1988. Automation of tissue culture manipulations in the final stages. Acta Horticulturae 227: 399-401.

Vanneste S, Friml J. 2009. Auxin: A trigger for change in plant development. Cell 136: 1005-1016.

Vasil IK, Vasil V. 1972. Totipotency and embryogenesis in plant cell and tissue cultures. *In Vitro* 8: 117-127.

Verdeil JL, Alemanno L, Niemenak N, Tranbarger TJ. 2007. Pluripotent versus totipotent plant stem cells: dependence versus autonomy. Trends in Plant Science 12: 245-252.

Visser E, Cohen JD, Barendse G, Blom C, Voesenek L. 1996. An Ethylene-Mediated Increase in Sensitivity to Auxin Induces Adventitious Root Formation in Flooded *Rumex Palustris Sm*. Plant Physiology 112: 1687-1692.

Vogel C, Abreu Rde S, Ko D, Le SY, Shapiro BA, Burns SC, Sandhu D, Boutz DR, Marcotte EM, Penalva LO. 2010. Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. Molecular Systems Biology 6: 400.

Vogel C, Marcotte EM. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics 13: 227-232.

Warrag EI, Lesney MS, Rockwood DL. 1990. Micropropagation of field-tested superior *Eucalyptus grandis* hybrids. New Forests 4: 67–79.

Watt MP. 2013. Genotypic-unspecific protocols for the commercial micropropagation of *Eucalyptus grandis* × *nitens* and *E. grandis* × *urophylla.* Turkish Journal of Agriculture and Forestry 38: 125-133.

Watt MP, Berjak P, Makhathini A, Blakeway F. 2003. *In vitro* field collection techniques for *Eucalyptus* micropropagation. Plant Cell Tissue and Organ Culture 75: 233 – 240.

Watt MP, Blakeway F, Cresswell CF, Herman BH. 1991. Somatic Embryogenesis in *Eucalyptus grandis*. South African forestry journal 157: 59-65.

Watt MP, Duncan EA, Ing M, Blakeway FC, Herman B. 1995. Field Performance of Micropropagated and Macropropagated *Eucalyptus* Hybrids. South African Forestry Journal 173: 17-21.

Watt MP, Mycock DJ, Blakeway F, Berjak P. 2000 a. Applications of *in vitro* methods to *Eucalyptus* germplasm conservation. South African forestry journal 187: 3-10.

Watt MP, Thokoane NL, Mycock DJ, Blakeway F. 2000 b. *In vitro* storage of *Eucalyptus grandis* germplasm under minimal growth conditions. Plant Cell Tissue and Organ Culture 61: 161-164.

Weatherhead MA, Burdon J, Henshaw GG. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. Zeitschrift Fur Pflanzenphysiologie 89: 141-147.

Wendling I, Brondani GE. 2014. Vegetative rescue and cutting propagation of *Araucaria angustifolia* (Bertol.) Kuntze1. Revista Arvore 39: 93-104.

Wendling I, Brondani GE, Dutra LF, Hansel FA. 2010. Mini-cuttings technique: a new *ex vitro* method for clonal propagation of sweetgum. New Forests 39: 343-353.

Wendling I, Warburton PM, Trueman SJ. 2015. Maturation in *Corymbia torelliana* × *C. citriodora* stock plants: Effects of pruning height on shoot production, adventitious rooting capacity, stem anatomy, and auxin and abscisic acid concentrations. Forests 6: 3763-3778.

Woo HR, Kim HJ, Lim PO, Nam HG. 2019. Leaf Senescence: Systems and Dynamics Aspects. Annual Review of Plant Biology 70: 347-376.

Xu T, Dai N, Chen J, Nagawa S, Cao M, Li H, Zhou Z, Chen X, De Rycke R, Rakusová H, Wang W, Jones AM, Friml J, Patterson SE, Bleecker AB, Yang Z. 2014. Cell surface *ABP1*-TMK auxin-sensing complex activates ROP GTPase signalling. Science 343: 1025-1028.

Yang JC, Chung JD, Chen ZZ. 1995. Vegetative propagation of adult *Eucalyptus grandis X urophylla* and comparison of growth between micropropagated plantlets and rooted cuttings. Plant Cell Reports 15: 170–173.

Yang ZB, Liu G, Liu J, Zhang B, Meng W, Muller B, Hayashi KI, Zhang X, Zhao Z, Smet ID, Ding Z. 2017. Synergistic action of auxin and cytokinin mediates aluminum-induced root growth inhibition in *Arabidopsis*. EMBO Reports 18: 1213-1230.

Yun JJ, Heisler LE, Hwang IIL, Wilkins O, Lau SK, Hyrcza M, Jayabalasingham B, Jin J, McLaurin JA, Tsao MS, Der SD. 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Research 34: 1-10.

Zheng L, Zhang L, Duhan K, Zhu ZP, Ye ZW, Gao QH. 2016. *YUCCA* type auxin biosynthesis genes encoding flavin monooxygenases in melon: Genome-wide identification and developmental expression analysis. South African Journal of Botany 102: 142-152.

Ziv M. 2005. Simple bioreactors for mass propagation of plants. Plant Cell Tissue and Organ Culture 81: 277-285.

Ziv M, Meir G, Halevy AH. 1983. Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. Plant Cell Tissue and Organ Culture 2: 55-56.

Appendix 1:

Plant growth regulator and supplement molar concentrations

Table A1 The respective concentrations of the various PGRs and supplements incorporated into the multiplication (Mt1-Mt5), elongation (Et1-Et4) and rooting (Rt1-Rt10) media represented in molar concentration.

Culture medium composition	Mt1	Mt2	Mt3	Mt4	Mt5	Et1	Et2	Et3	Et4	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7	Rt8	Rt9	Rt10
PGR																			
IAA (µM)	0.57	0.57	0.57	0.57	0.57	2.85	2.85	2.85	2.85	0.57	2.85	0.57	2.85	–	–	–	2.85	2.85	–
IBA (µM)	–	–	–	–	–	–	–	–	–	–	–	–	–	0.49	2.46	–	–	–	–
NAA (µM)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.54	–	–	–
GR24[1] (µM)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.1	–
BAP (µM)	0.22	1.11	0.44	–	2.22	–	–	0.22	–	–	–	–	–	–	–	–	–	–	–
Kinetin (µM)	2.32	1.16	0.47	–	0.47	–	0.23	–	–	–	–	–	–	–	–	–	–	–	–
Meta-topolin (µM)	–	–	–	2.07	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–
Supplement																			
MS (g ℓ^{-1})	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Vitamin B5 (µM)	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Biotin (µM)	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41
Sucrose (mM)	58.4	58.4	58.4	58.4	58.4	58.4	58.4	58.4	58.4	58.4	58.4	43.8	43.8	43.8	43.8	43.8	43.8	43.8	43.8
BC2O4[2] (g ℓ^{-1})	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.1	–	–
Activated charcoal (M)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.83

[1] Synthetic strigolactone. [2] Organic biostimulant synthesized from citrus extracts and organic acids.

Appendix 2:

Change in explant length and bud number for multiplication

Table A2 The mean ($\bar{x}$) change in explant length and bud number ± standard error (in parentheses), for clones 1, 2 and 3, following a 4 – week growth period, on five media treatments (Mt1-Mt5), repeated in triplicate, is represented. The lower-case letters denote significant differences between the treatments and the upper-case letters indicate significant differences between the clones, analysed via Tukey's HSD with a 95% confidence interval ($p \leq 0.05$).

Clone	Mt1		Mt2		Mt3		Mt4		Mt5	
	$\bar{x}$ Length (cm)	$\bar{x}$ Bud no.	$\bar{x}$ Length (cm)	$\bar{x}$ Bud no.	$\bar{x}$ Length (cm)	$\bar{x}$ Bud no.	$\bar{x}$ Length (cm)	$\bar{x}$ Bud no.	$\bar{x}$ Length (cm)	$\bar{x}$ Bud no.
1	0.40 ± (0.04) aA	17.25 ± (1.44) aA	0.45 ± (0.04) adB	26.72 ± (1.34) bB	0.87 ± (0.08) bC	31.06 ± (1.48) bC	1.23 ± (0.08) cDE	59.11 ± (2.97) cDEF	0.65 ± (0.05) bdF	51.72 ± (2.42) cEFG
2	1.69 ± (0.09) abG	36.25 ± (1.34) aCH	1.36 ± (0.09) acDI	65.11 ± (2.37) bDI	1.51 ± (0.09) abcD	65.14 ± (2.45) bDI	1.81 ± (0.08) bH	62.31 ± (2.75) bDE	1.20 ± (0.09) cDE	67.97 ± (2.74) bD
3	1.11 ± (0.06) aCEI	31.03 ± (1.25) aBC	0.74 ± (0.05) bCF	45.14 ± (1.56) bGH	0.92 ± (0.06) abCEF	50.5 ± (2.4) bcF	1.20 ± (0.07) aD	58.06 ± (3) cDE	0.97 ± (0.07) abCE	54.97 ± (2.19) **bc**EI

Appendix 3:

Change in explant length for elongation

Table A3 The mean (x̄) change in explant length ± standard error (in parentheses), for clones 1, 2 and 3, following a 4 – week growth period, on four media treatments (Et1-Et4), repeated in triplicate, is represented. The lower-case letters denote significant differences between the treatments and the upper-case letters indicate significant differences between the clones, analysed via Tukey's HSD with a 95% confidence interval ($p \leq 0.05$).

Clone	Et1	Et2	Et3	Et4
	x̄ Length (cm)	**x̄ Length (cm)**	**x̄ Length (cm)**	**x̄ Length (cm)**
1	0.882 ± (0.09) aA	0.488 ± (0.06) bB	2.127 ± (0.06) cCF	2.629 ± (0.07) cDF
2	1.396 ± (0.12) aE	2.596 ± (0.13) bcDF	2.221 ± (0.1) bCF	2.935 ± (0.09) cD
3	0.971 ± (0.06) aAE	0.474 ± (0.04) bB	1.766 ± (0.09) cC	2.968 ± (0.09) dD

Appendix 4:

Root length for the rooting treatments 1-10

Table A4 Mean (x̄) values plus/minus standard error (in parentheses) for root length following a 4 – week growth period for clones 1, 2 and 3 grown on ten different rooting media (Rt1-Rt10). Letters indicate significant differences between the treatments, for a given clone, determined by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

Clone	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7	Rt8	Rt9	Rt10
	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)	x̄ Length (cm)
1	0.03 ± (0.02) a	0.13 ± (0.06) ab	1.15 ± (0.38) bc	1.99 ± (0.6) c	2.54 ± (0.72) c	0.29 ± (0.15) ab	0 ± (0) a	1.54 ± (0.45) c	2.31 ± (0.74) c	0 ± (0) a
2	0.03 ± (0.01) a	0.06 ± (0.03) a	2.93 ± (1.07) ab	3.32 ± (0.89) bd	11.4 ± (2.31) c	4.39 ± (1.47) d	0.51 ± (0.35) a	1.21 ± (0.76) a	4.45 ± (1.11) d	0.47 ± (0.35) a
3	0.02 ± (0.01) ab	0.02 ± (0.01) ab	0.90 ± (0.3) bc	1.47 ± (0.39) c	0.73 ± (0.33) ac	7.79 ± (2.2) d	0 ± (0) a	1.95 ± (1.02) bc	2.53 ± (0.77) c	1.77 ± (0.85) bc

Appendix 5:

Root number for the rooting treatments 1-10

Table A5 Mean (x̄) values plus/minus standard error (in parentheses) for the root number following a 4 – week growth period for clones 1, 2 and 3 grown on ten different rooting media (Rt1-Rt10). Letters indicate significant differences between the treatments, for a given clone, determined by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

Clone	Rt1	Rt2	Rt3	Rt4	Rt5	Rt6	Rt7	Rt8	Rt9	Rt10
	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number	x̄ Number
1	0.11 ± (0.07) ab	0.14 ± (0.07) ac	0.33 ± (0.09) bcd	0.81 ± (0.21) d	0.97 ± (0.23) d	0.3 ± (0.17) ac	0 ± (0) a	0.5 ± (0.13) d	0.5 ± (0.16) cd	0 ± (0) a
2	0.08 ± (0.04) a	0.11 ± (0.05) a	0.31 ± (0.10) abe	0.53 ± (0.15) be	2.14 ± (0.41) c	1.5 ± (0.38) cd	0.08 ± (0.06) a	0.17 ± (0.07) ab	0.58 ± (0.13) de	0.14 ± (0.07) a
3	0.08 ± (0.04) ab	0.06 ± (0.04) ab	0.25 ± (0.07) bc	0.53 ± (0.14) c	0.36 ± (0.16) abc	2.53 ± (0.62) d	0 ± (0) a	0.22 ± (0.11) abe	0.53 ± (0.16) ce	0.25 ± (0.1) abc

Appendix 6:

Root length and number for the top three rooting treatments 1-10, repeated in triplicate

Table A6 Mean (x̄) values plus/minus standard error (in parentheses) for root length and number following a 4-week growth period for clones 1, 2 and 3 grown on Rt4, Rt6 and Rt9. All treatments were replicated in triplicate. The lower-case letters indicate significant differences between the treatments, and the upper-case letters denote statistical significance between the clones, as determined by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with a 95% confidence interval ($p \leq 0.05$).

Clone	Rt4		Rt6		Rt9	
	x̄ Length (cm)	x̄ Root no.	x̄ Length (cm)	x̄ Root no.	x̄ Length (cm)	x̄ Root no.
1	1.66 ± (0.63) abA	0.65 ± (0.18) aAB	0.47 ± (0.2) aB	0.33 ± (0.14) aB	3.24 ± (1.01) bAC	0.69 ± (0.18) aAB
2	2.74 ± (0.82) aA	0.5 ± (0.14) aB	4.25 ± (1.26) aC	1.29 ± (0.35) bA	4.65 ± (1.23) aC	0.68 ± (0.15) abAC
3	1.25 ± (0.37) aAB	0.48 ± (0.13) aBC	5.02 ± (1.49) bC	1.44 ± (0.37) bA	3.27 ± (0.95) abAC	0.66 ± (0.19) abAB